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14. ABSTRACT
This progress report describes the assays, protocols and results related to a collaborative research initiative between Dr. Mann's research laboratories (coagulation research laboratory and Antibody Core Facility, Department of Biochemistry, University of Vermont, Burlington, Vermont) and Dr. Park's clinical research involving trauma and acutely burned patients (U. S. Army Institute of Surgical research, Fort Sam Houston, Texas). The major areas of research involved assessment of the role of soluble and membrane-bound tissue factor (TF) in the overall responses of trauma and burned patients to interventions and to determine whether TF concentrations in blood and/or on cell over time can predict the risks for developing Multi-Organ Dysfunction syndrome (MODS) or Adult Respiratory Distress Syndrome (ARDS). Dr. Mann's laboratory has determined the TF antigen concentrations in plasmas from 149 trauma and burned patients. Each patient had several plasma samples that had been collected at various times following hospitalization. These samples include baseline (the day of hospitalization), day 1, day 3, day 5, day 7, and plasma samples drawn in between hospitalization days and the discharge days (in most cases up to 42 days). The total numbers of plasmas tested were 514. For each sample duplicate determinations were performed. The TF activity was also determined for 514 plasmas and when TF activity was detected, anti-TF monoclonal antibody (mAb) was used to verify the observation. If active TF was found in plasma, anti-TF mAb should inhibit the activity. These data were provided to Dr. Park for further analyses of the correlation of the TF antigen and activity with clinical data obtained by Dr. Park's team.

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Parhami-Seren B, Butenas S, Krudysz-Amblo J and **Mann KG:** Immunologic quantitation of tissue factors. Journal of Thrombosis and Haemostasis 4: 1747-1755, 2006.

Butenas S, Bouchard B, Brummel-Ziedins K, Parhami-Seren B and **Mann KG**: Tissue factor activity in whole blood. Blood. 7:2764-2770, 2005.

INTRODUCTION

This progress report describes the assays, protocols and results related to a collaborative research initiative between Dr. Mann's research laboratories (coagulation research laboratory and Antibody Core Facility, Department of Biochemistry, University of Vermont, Burlington, Vermont) and Dr. Park's clinical research involving trauma and acutely burned patients (U. S. Army Institute of Surgical research, Fort Sam Houston, Texas).

The major areas of research involved assessment of the role of soluble and membrane-bound tissue factor (TF) in the overall responses of trauma and burned patients to interventions and to determine whether TF concentrations in blood and/or on cell over time can predict the risks for developing Multi-Organ Dysfunction syndrome (MODS) or Adult Respiratory Distress Syndrome (ARDS).

Dr. Park has provided Dr. Mann's laboratory with 514 plasma samples from trauma and burned patients for TF analyses. Dr. Mann's laboratory has developed novel high throughput assays for the detection of TF antigen and activity in plasma (Butenas and Mann 2004; Parhami-Seren, Butenas et al. 2006) and on blood cells. These assays include a highly sensitive and specific fluorescence-based immunoassay for quantifying soluble, intracellular and membrane-bound TF and an activity-based assay by which picomolar concentrations of soluble and membrane-bound TF can be readily detected in plasma and/or on cells. These plasma samples were tested for the presence of TF antigen and for TF activity. The presence of TF activity was confirmed using a highly specific anti-human TF monoclonal antibody (anti-TF mAb) which specifically inhibits TF activity.

Results:

1. Introduction to the assays and tools that will be used to study TF in trauma and acutely burned patients

In preparation for the above research initiatives between Dr. Park and Dr. mann, we have developed and validated assays and experimental protocols as follows.

Description of fluorescence immunoassay format and Luminex Multi-Analyte Platform.

Fluorescence-based immunoassay of tissue factor (TF).

Following extensive analyses of mAbs by ELISA, two mAbs anti-TF-5 as capture mAb and anti-TF-48 as detection mAb were selected for fluorescence-based immunoassay (Parhami-Seren, Butenas et al. 2006). We have developed and evaluated highly sensitive and specific fluorescence-based TF immunoassay that can universally measure tissue factor (TF) antigen concentrations in recombinant products, natural sources, cell lysates and cell membrane and plasma. Further analyses, of human tissue factor (TF) proteins isolated from natural sources such as placenta and monocytes and those produced *ex vivo* via engineering methods in yeast, bacteria and insect cells, demonstrated that there are significant structural heterogeneity among these proteins thus emphasizing on the importance of having monoclonal antibodies (mAbs) and immunoassay that can recognize TFs from various sources.

One advantage of integrating fluorescence immunoassays into LMAP technology is that specific and non-specific binding can be determined simultaneously in one reaction well. This feature of fluorescence assay is particularly important when assays are performed on plasma since heterophilic Abs or other cross-reactive components of plasma could skew the results (Levinson 1992; Kaplan and Levinson 1999; Kricka 1999; Maple, Lathrop et al. 2004). Thus anti-TF-5 mAb was coupled to beads with identification #035 and an isotype matched mAb with specificity irrelevant to TF was coupled to beads 044. Beads were mixed and reacted with TF-immunodepleted plasma which was spiked with various concentrations of recombinant (r)TF 1-263, rTF 1-242, rTF 1-218 and placental TF. Binding was probed with biotinylated anti-TF-48 mAb and PE-streptavidin.

Fig. 1

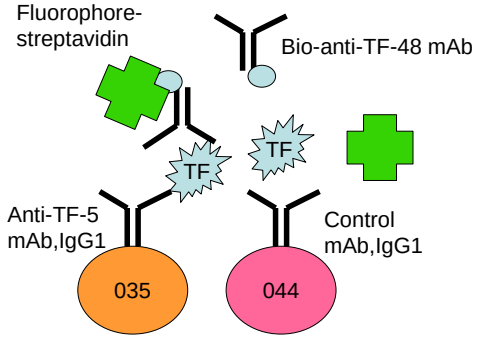


Fig. 2

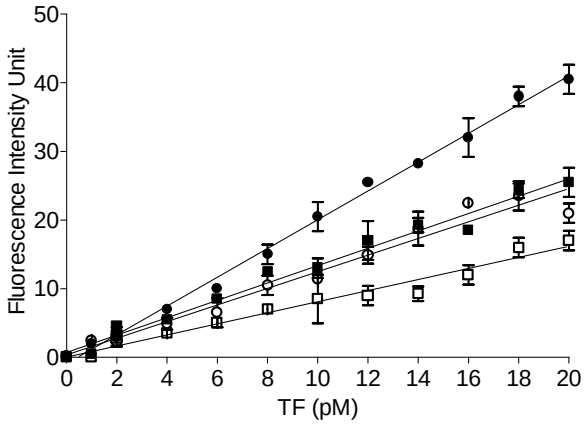
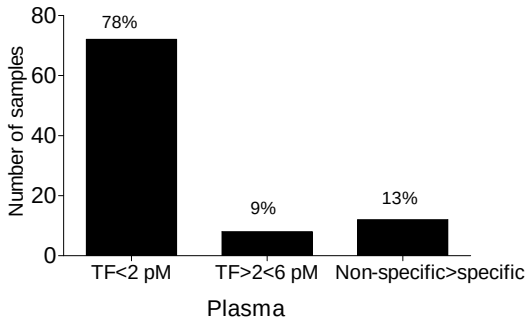


Fig. 1 is the schematic presentation of the TF assay and Fig. 2 illustrates TF concentration dependence of the assay (Parhami-Seren, Butenas et al. 2006). TF was spiked in TF-immunodepleted plasma and concentrations as low as 1-2 pM could be reliably detected. *○ rTF 1-218, ■ rTF 1-242, ● rTF1-263, □ placenta TF*

We evaluated this TF assay by using plasma from healthy individuals (n=92) and found that the concentration of TF antigen in plasma of 72 individuals (78%) was below 2 pM (reliable detection limits of the assay). TF antigen levels between 2.3-5.5 pM could be detected in 8 individuals (9%) and in 13% (12 plasmas), the non-specific signal was

higher than specific signal (Fig. 3). Therefore, TF levels could not be determined in these plasmas (Parhami-Seren, Butenas et al. 2006).

Fig. 3. (TF antigen concentrations and ranges in plasma of Healthy donors, n=92)

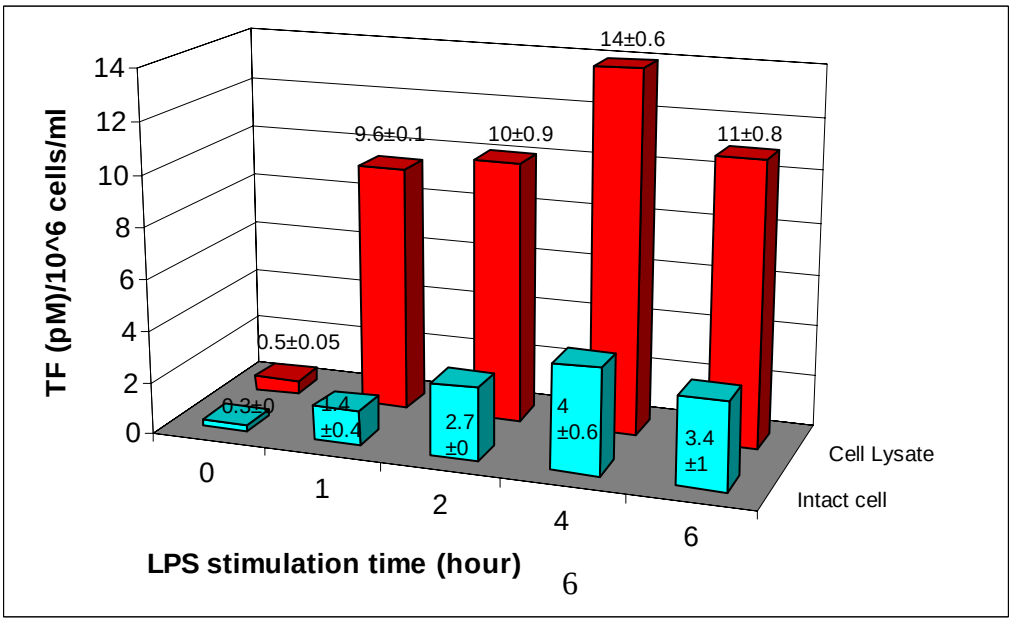


Our data conclude that there is quantifiable TF-like antigen in plasma of some healthy individuals. These assays are thus important tools by which one could more precisely determine whether there is a relationship between elevated circulating TF antigen concentration in blood and a particular disorder.

TF concentrations on cells and in cell lysates:

Unstimulated and lipopolysacharride (LPS) stimulated THP1 human monocytic cell line was utilized as model cells to analyze TF expression, quantitation and functional properties. Fig. 4 illustrates a comparison between the concentration of membrane-bound TF (intact cells) and total concentration of TF (lysate, membrane bound and intracellular) in monocytes as a function of time following stimulation which was determined using FLI. Concentrations were determined from the calibration curve for recombinant (r) TF 1-263 similar to that shown in Fig. 2.

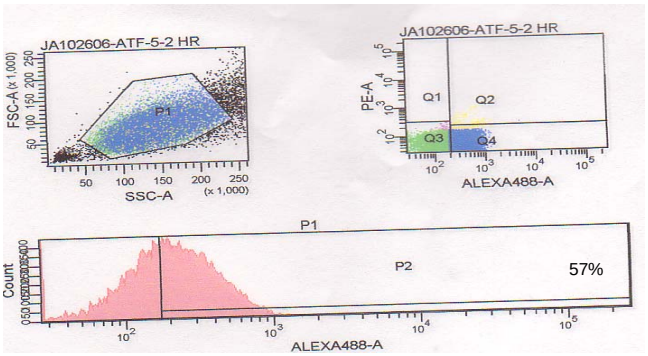
Fig. 4

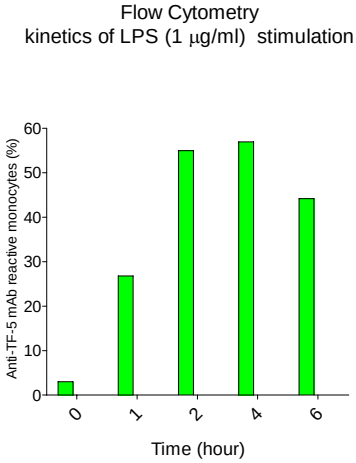


Conclusions from the above experiments include the observation that the TF expression and concentration reached maximum after 4 hours of stimulation with 1 μg/ml LPS. In addition, at maximum expression, only 28% of the total cellular TF reservoir is displayed on the cell surface. Time-dependency of TF display on the cell membrane was confirmed by flow cytometric analysis of the cells from the same experiment) using Alexa 488-labeled anti-TF-5 (Fig. 5). Flow cytometric analyses indicated that while only 3% of the unstimulated cells were positive for TF, after 4 hr LPS stimulation, 60% of the cells reacted with anti-TF mAb. Similar to the data presented in Fig. 4, there was quantitative and time dependent TF expression ranging from 27% TF positive cells at 1 hr, reaching maximum at 4 hr and declining to 44% after 6 hr exposure to LPS. In addition, flow cytometry results indicated that 40% of the monocytes do not display TF on their membrane (Fig. 5).

Fig. 5

Anti-TF mAb reactive cells (4 hr LPS stimulated cells)





More detailed analysis of TF expression by confocal microscopy confirmed the presence of intracellular TF in 100% of the cells though there were significant differences in signal intensity among these cells (Fig. 6) consistent with the presence of heterogeneity among monocytes.

Concentration of the membrane-bound procoagulant TF was assessed using clot time and FXa generation. In the clotting assay where unstimulated and LPS stimulated monocytes were used, there was a time-dependent increase in TF procoagulant activity which reached maximum after 4 hrs (Table 1) consistent with the results of the FLI and flow cytometry. The data regarding membrane-bound TF activity in clotting assay and chromogenic assay of FXa generation is summarized in Table 1 and compared to TF antigen concentration by FLI. TF concentrations determined by FLI or by clotting assay were calculated from their

corresponding calibration curves in which increasing molar concentrations of rTF 1-263 had been used. The data presented in Table 1 confirm that the membrane-bound TF exhibits procoagulant activity by initiating TF-dependent coagulation pro

Fig. 6

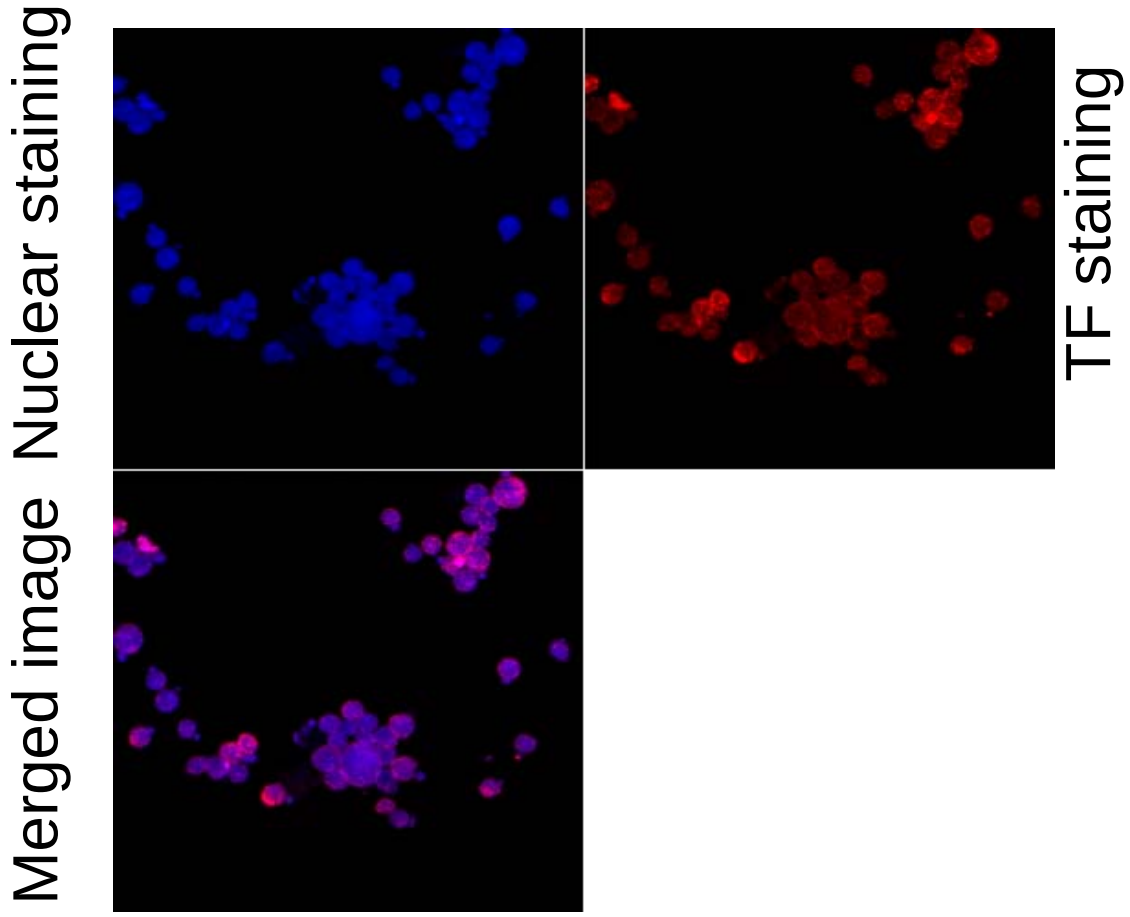


Table 1. Comparison of TF antigen and activity on intact monocytes

LPS stimulation (hr)	pM TF/10^6 cells/ml (FLI)	pM TF/10^6 cells/ml (clot time)	pM FXa/min/10^6 cells/ml (chromogenic)
0	0.3±0.0	9.8±0.5	250
1	1.4±0.4	39±0.0	1059
2	2.7±0.0	88±0.6	1481
4	4.0±0.6	95±1.8	1643
6	3.4±1.0	87±1.3	2778

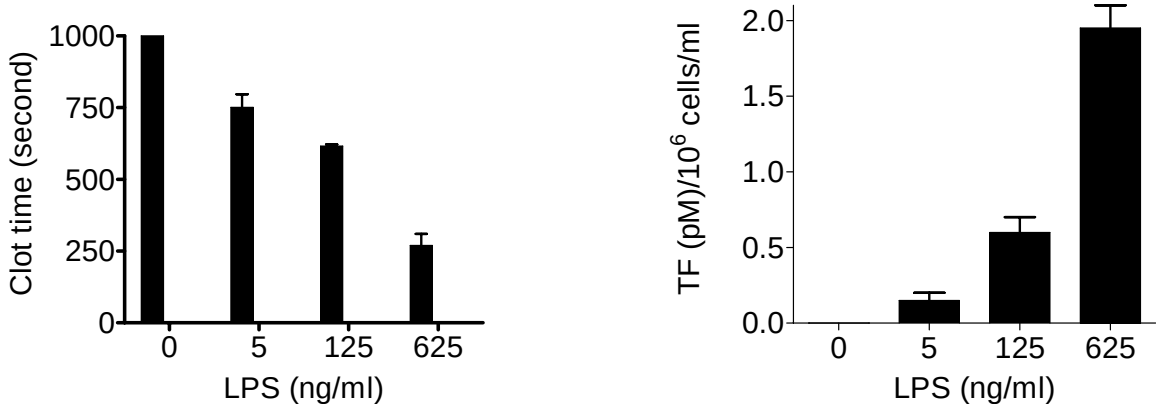
These studies set the platform for more through analyses of the TF expression and function by human blood monocytes in trauma and burned patients.

Studies involving analyses of TF on human white blood cells

We have determined an optimum LPS concentration at which TF antigen can be detected unambiguously on the white blood cells. Contact pathway inhibited blood (using corn trypsin inhibitor, CTI) from a healthy individual was obtained and puffy coat was separated (unfractionated cells). Unstimulated and LPS stimulated cells were analyzed for TF expression using FLI, clotting assay and flow cytometry. Fig. 7 illustrates a comparison between TF concentration and procoagulant activity of the unfractionated human white blood cells. There was an increase in TF expression as a function of increasing concentration of LPS. TF antigen levels reached to 2±0.2 pM upon stimulation with 625 ng/ml LPS for 2 hrs. Similarly, procoagulant activity of cells was higher at 625 ng/ml LPS. These patterns of response are consistent with those of the monocytic cell line (Fig. 4 and Table 1).

Flow cytometric analyses was performed on the same cell population that were stimulated with 625 ng/ml LPS and is illustrated in Fig. 8. Flow cytomery results indicated that 14.8% of the cells expressed TF but 7.8% were positive for CD14 (a monocyte-specific marker, data not shown).

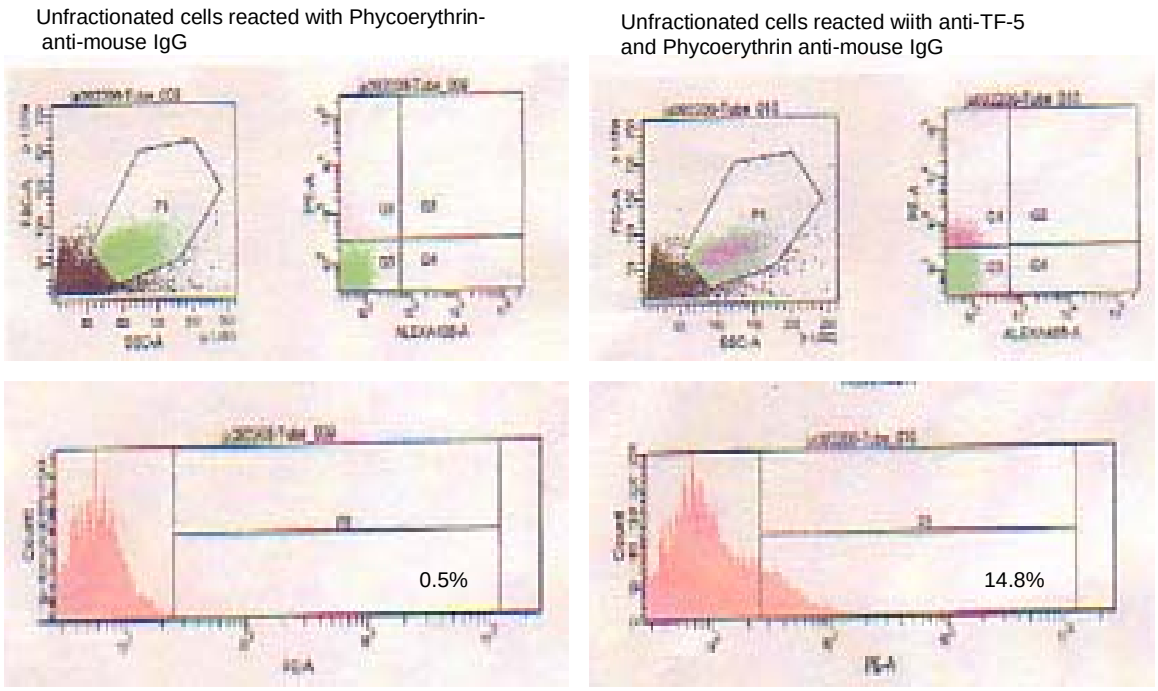
Fig. 7



CONCLUSIONS

These experiments demonstrate the availability of tools and feasibility of the experimental protocols for the analyses of TF expression and procoagulant activity in plasma and on the white blood cells. Thus we will determine whether the time course changes in TF concentration and procoagulant activity in plasma and/or on cells shows association with the development of MODS or ARDS.

Fig. 8



2. Studies involvingTF antigen concentration and procoagulant activity determination in plasma of trauma and acutely burned patients

The first part of this research initiative was to determine TF antigen concentration and procoagulant activity in the plasma samples (n=514) drawn from 149 trauma and burned patients over time.

Table 2 exhibits a complete TF antigen concentration and procoagulant activity among patients with trauma and burned injuries.

Table 2. TF concentrations (pM) by fluorescence-based immunoassay (FLI) and by clot time (CT).

ID	TF (pM)	Base line	Day 1	Day 2	Day 3	Day 5	Day 7	Day 14	Day 15	Day 21	Day 22	Day 28	Day 29	Day 30	Day 35	Day 36	Day 42	Day 49	Day 56	discharge
	FLI	1.4+/-0.3	1.4+/-0.3	0	1.4+/-0.3	1.2+/-0	1.4+/-0.3													
26	CT	0.95	0.68	0	0	0	0													
	FLI	0	3.1+/-0	1.5+/-0.8	1.6+/-0	1.8+/-0.8	1.2+/-0													
27	CT	0	0	0	0.56	0	0													
	FLI	0.6+/-0	1.2+/-0	1.1+/-1.5	0.6+/-0.8	0.8+/-0.2	0.3+/-0.4	1.0+/-0.6		0		0.6+/-0.4			1.3+/-0.1		0.9+/-0.4	0.2+/-0.2	0.9+/-0.4	
28	CT	0	0	0	0	0	0	0		0		0			0		0	0	0	
	FLI	2.1+/-0.7	3.1+/-0	2.5+/-0.6	2.1+/-0	1.7+/-0.6	3.1+/-0.7													
29	CT	0	0	0		0	0													
	FLI	1.1+/-0.7	0.6+/-0.8	0.8+/-0.2	1.1+/-0.7	1.9+/-0.4	0.6+/-0.8				1.9+/-2.2			1.6+/-0						
30	CT	0	0	0	0	0	0				0			0						
	FLI	0.9+/-0.4	1.3+/-0.1	0.3+/-0.4	0	0	0.9+/-0.4													
31	CT	0	0	0	0	0	0													
	FLI	0.4+/-0.3	0.1+/-0.2	0	0.6+/-0	0	0													
32	CT	0	0	0	0	0	0													
	FLI	0.3+/-0.4	0	0.3+/-0.4	0															
33	CT	0	0	0	0															
	FLI	0.3+/-0.4	0	0.9+/-0.4	0															
34	CT	0	0	0	0															
	FLI	0.3+/-0.4	0.2+/-0.2	0	0.2+/-0.2															
35	CT	0	0	0	0															
	FLI	0																		
36	CT	1.05																		
	FLI	0	0	0.8+/-1.1	0.7+/-1.0	0.5+/-0.6	0.8+/-1.1		0		0		0			0.3+/-0.4				
37	CT	0	0	0	0	0	0		0		0		0			0				
	FLI	0	0																	
49	CT	0	0																	
	FLI	0.6+/-0.8	0	0.8+/-0.2	0.3+/-0.4	0														
50	CT	0	0	0	0	0.59														
	FLI	1.1+/-0.7	0.6+/-0.8	1.6+/-0																
51	CT	0	0	0.5																
	FLI	0																		
52	CT	0																		
	FLI	0	0	0	0	0	0													
53	CT	0	0.5	0	0	0	0													
	FLI	0	0.5+/-0.6																	
54	CT	0	0.91																	
	FLI	2.1+/-1.3	1.0+/-0.2	0	0.2+/-0.3	1.1+/-0.2	1.1+/-0.5	0.9+/-0.8		1.4+/-0.8		1.8+/-0.3								1.5+/-1.0
55	CT	0	0	0	0	0	0	0		0		0								0
	FLI	0.3+/-0.3	0.1+/-0.2	0.4+/-0.7	0.9+/-0.6	1.3+/-0.2	1.0+/-0.3													
56	CT	0	0	0	0	0	0													
	FLI	0	0	0	0	0	0													
57	CT	0	0	0	0	0	0													
	FLI	0	0.2+/-0.3	0	0.6+/-0.6	0.4+/-0.7														
58	CT	0	0	0	0															
	FLI	0																		
59	CT	0																		
	FLI	1.1+/-0.5	0.6+/-0.6	1.6+/-0.5	1.6+/-0.5	3.2+/-0.6	5.0+/-0.5													3.4+/-0.3
60	CT	0	0	0	0		0													0
	FLI	0.6+/-0.6	1.3+/-0.6	3.7+/-0.5	4.1+/-0.8	3.2+/-0.2														
61	CT	0	0	0	0	0														
	FLI	0.6+/-0.6	0.3+/-0.5	0.4+/-0.7																
62	CT	0.59	0	0																
	FLI	0.7+/-0.8	1.4+/-0.6	0.6+/-0.6	1.0+/-0.3	0.6+/-0.6														
63	CT	0	0	0	0	0														
	FLI	0.2+/-0.3	0.3+/-0.5																	
64	CT	0	0																	
	FLI	0.8+/-0.7	1.3+/-0.1	1.8+/-0.5	1.3+/-0.6	1.1+/-0.5	1.1+/-0.5													
65	CT	0	0	0	0	0	0													
	FLI	6.8+/-0.8	1.4+/-0.2	2.1+/-0.5																
66	CT	0	0	0																
67	FLI	0.4+/-0.7	0.1+/-0.2	0.4+/-0.3	0.2+/-0.3	0.2+/-0.3														
ATF	CT	0.79	0	0	0.68	0														
68	FLI	0.2+/-0.3				1.5+/-0.2	1.9+/-0.6													
ATF	CT	0				0	0													
69	FLI	0	0	0	0	0	0													
ATF	CT	0	0	0	0	0	0													
70	FLI	1.1+/-0.7	0.9+/-0.8	1.3+/-0.2	0.6+/-0.6	1.0+/-0.3	0.3+/-0.5													
ATF	CT	7.75	0	0	0	0	0													
71	FLI	1.0+/-0.3	0.1+/-0.2	0.6+/-0.6																
ATF	CT	0	0	0																
72	FLI	0	0	0.2+/-0.3	0.6+/-0.6	0.9+/-0.8	0.8+/-0.3													
ATF	CT	0	0	0	0	0	1.08													
					Volunteer ID															
			12ATF	13ATF	14ATF	15ATF	16ATF	17ATF	18ATF	19ATF	20ATF									
	FLI	0.2+/-0.3	0.9+/-0.6	1.3+/-0.2	1.5+/-0.5	3.8+/-0.3	0.8+/-0.7	3.8+/-0.3	4.1+/-0.5	0										
	CT	0.71	0	0	0.56	0	0	0	0	0										

ID	TF(pM)	Base line	Day 1	Day 2	Day 3	Day 5	Day 7	Day 30	Day 39	Day 42
	FLI	1.7 +/-0.8	1.6 +/- 0.1	0.4 +/- 0.2	1.1 +/- 0	1.1 +/- 0	1.4 +/- 0.7			
73	CT	0	0	0	0	0	0			
	FLI	14.4 +/- 1.7	3.7 +/- 1.6	1.8 +/- 2.5	2.9 +/- 1.5	2.3 +/- 2.4				
74	CT	0	0	0	0	0	0			
	FLI	0.7 +/- 0.2	0.3 +/- 0.4	0.3 +/- 0.4	0.1 +/- 0.2	0.1 +/- 0.2	3.2 +/- 1.3			
75	CT	0	0	0	0	<1	0			
	FLI	0.2 +/- 0.3	1.2 +/- 0.1	0.1 +/- 0	0.5 +/- 0.1	0.1 +/- 0.1	0.3 +/- 0.4			
76	CT	<1	0	0	<1	0	0			
	FLI	1.1 +/- 0.4	0.9 +/- 0.4	0.9 +/- 0.1	2.1 +/- 3.0	0.9 +/- 0.4	0.4 +/- 0.6			
77	CT	0	0	0	0	<1	<1			
	FLI	1.6 +/- 0.3	2.6 +/- 0.5	1.9 +/- 0.1	1.6 +/- 0.1	0.9 +/- 0.5	1.5 +/- 0.5			
78	CT	<1	<1	0	<1	0	0			
	FLI	0.8 +/- 0.3	0.6 +/- 0.5	0.1 +/- 0.2	0.9 +/- 0	0.1 +/- 0.2	0.8 +/- 0.1			
79	CT	0	<1	0	<1	<1	2.3			
	FLI	3.0 +/- 0.6	1.7 +/- 1.0	0.8 +/- 0.1	1.6 +/- 0.6					
80	CT	0	<1	0	<1					
	FLI	0.9 +/- 0.1	1.1 +/- 0	1.3 +/- 1.4	0.8 +/- 0.1	2.7 +/- 0.2			1.8 +/- 1.4	
81	CT	<1	0	<1	0	0			0	

ID		Base line	Day 1	Day 2	Day 3	Day 5	Day 7	Day 30	Day 39	Day 42
82	FLI	0.9 +/- 0.3	0.8 +/- 0.7	0	0	0.4 +/-0.5	0.2 +/- 0.3	0.1 +/- 0.2		
	CT	0	0	0	0	*0*	*0*	*0*		
	FLI	1.1 +/- 0.4	1.0 +/- 0.2	0.9 +/- 0.5	0.8 +/- 0.1	1.4 +/- 0.4	1.3 +/- 0.6			
83	CT	0	0	0	0	0	0			
	FLI	1.8 +/- 0.1	1.1 +/- 0.8							
84	CT	0	0							
	FLI	0.9 +/- 0.1	0.6 +/- 0.3							
85	CT	0	<1							
	FLI	0.4 +/- 0.6	0.8 +/- 0.1							
86	CT	<1	0							
	FLI	1.1 +/- 0.3	1.1 +/- 0	1.1 +/- 0.1	1.5 +/- 0.3		0.4 +/- 0.3			
87	CT	0	0	0	<1		0			
	FLI	0.1 +/- 0.1	0.4 +/- 0.2	0.9 +/- 0.3	0.8 +/- 0.1	0.7 +/- 0.6	0			191 +/- 3.1
97	CT	0	0	0	0	0	0			0
	FLI	1.5 +/- 0.1	1.6 +/- 0.1	1.3 +/- 0.6	1.1 +/- 0	0.6 +/- 0	0.1 +/- 0.2			
98	CT	0	0	<1	0	0	0			
	FLI	33 +/- 4.8	11 +/- 0.1							
99	CT	0	<1							
	FLI	1.0 +/- 0.6	0	0	1.0 +/- 0.8	0.6 +/- 0.8				
100	CT	0	<1	0	0	0				

ID		Base line	Day 1	Day 2	Day 3	Day 5	Day 7	Day 30	Day 39	Day 42
	FLI	14.6 +/- 2	5.0 +/- 0.5	2.4 +/- 1.0	1.3 +/- 0.6	1.7 +/- 1.0	1.4 +/- 0			
101	CT	0	0	0	0	0	0			
	FLI	0.9 +/- 0.3	1.3 +/- 0.2							
102	CT	no sample	0							
	FLI	1.1 +/- 0.3	0.4 +/- 0.2	0.6 +/- 0.2	0.6 +/- 0.1	1.2 +/- 0.5				
103	CT	<1	0	0	0	<1				
	FLI	0.1 +/- 0.1	0.7 +/- 0.2	0.1 +/- 0.2	0.6 +/- 0					
104	CT	<1	0	0	0					
	FLI	1.3 +/- 0.2	0.9 +/- 0.4	0.4 +/- 0.4	0.9 +/- 0.7	0.5 +/- 0.7	1.2 +/- 0.5			
105	CT	<1	0	1.8	no sample	0	0			
	FLI	0.2 +/- 0.3	1.0 +/- 0.2	0.2 +/- 0.1	0.5 +/- 0.1	0.1 +/- 0.2	0.5 +/- 0.7			
106	CT	0		0	0	0	0			
	FLI	2.4 +/- 0.6	2.1 +/- 0.3	1.8 +/- 1.1	1.1 +/- 0.1	1.3 +/- 0.2	0.9 +/- 0.1			
107	CT	<1	<1	0	<1	0	0			
	FLI	2.4 +/- 0.6	1.0 +/- 0.6							
108	CT	0	0							
	FLI	0.6 +/- 0.4	0.9 +/- 0	1.0 +/- 0.4						
109	CT	0	0	<1						
	FLI	0.5 +/- 0.3	0.7 +/- 0.2	0.4 +/- 0.3	1.1 +/- 0.5					
110	CT	<1	0	0	0					
ID		Base line	Day 1	Day 2	Day 3	Day 5	Day 7	Day 30	Day 39	Day 42
	FLI	0.7 +/- 0.2	0.8 +/- 0.1	1.4 +/- 0.1	0.3 +/- 0.4					
111	CT	0	<1	0	0					
	FLI	1.4 +/- 0.8								
112	CT	0								
	FLI	1.0 +/- 0.2	1.1 +/- 0.4							
113	CT	0	0							
	FLI	0.9 +/- 0.4	1.6 +/- 0.3	1.1 +/- 0	0.6 +/- 0.7	0	0			
114	CT	0	0	0	0	0	0			
	FLI	0.9 +/- 0.3	1.4 +/- 0.4	1.6+/-0.2	1.1 +/- 0.1	2.6 +/- 0	0.3 +/- 0			
115	CT	0	0	0	0	0	0			
	FLI	0.9 +/- 0	0.1 +/- 0.1		1.2 +/- 0.3	0.4 +/- 0.6	1.2 +/- 0.5			
116	CT	0	0		0	<1	0			
	FLI	1.4 +/- 0.4	1.3 +/- 0.6	0.6 +/- 0.4	0.6 +/- 0	0.7 +/- 0.2	0.6 +/- 0.8			
117	CT	0	0	0	0	0	0			
	FLI	1.3 +/- 0.2		1.7 +/- 0	0.9 +/- 0.1	1.1 +/- 0.5				
118	CT	0		0	0	0				
	FLI	1.0 +/- 0.2		0.1 +/- 0.2						
119	CT	0		0						
	FLI	2.2 +/- 0.1	1.5 +/- 0.1	0.6 +/- 0.4	1.3 +/- 0.2	0.4 +/- 0.5	0.4 +/- 0.6			
120	CT	0	0	0	0	0	0			

ID		Base line	Day 1	Day 2	Day 3	Day 5	Day 7	Day 30	Day 39	Day 42
	FLI	0	0.6 +/- 0	1.1 +/- 0.5	0.1 +/- 0.2	0	0			
121	CT	0	0	0	0	0	0			
	FLI	4.1 +/- 0.4	0.8 +/- 0.3	0	0					
122	CT	<1	0	0	0					
	FLI	0	0	0	0	0	0.1 +/- 0.2			
123	CT	0	0	0	0	0	0			
	FLI	0.7 +/- 0	0.4 +/- 0.3	0	0	0	0.1 +/- 0.2			
124	CT	0	0	0	0	0	0			
	FLI	2.1+/-0.7	0.9 +/- 0.5	0.1 +/- 0.2						
125	CT	<1	<1	no sample						
	FLI	0	0	0.6 +/- 0.9						
126	CT	<1	0	*0*						
	FLI	0.6 +/- 0.4								
127	CT	0								
	FLI	13.5+/-4.6	0.7 +/- 1.0	0.6 +/- 0.4	0	0	3.6+/-0.6			
128	CT	0	*no sample*	1.4	0	0	<1			
	FLI	0.6 +/- 0.4	1.3 +/- 0.2	0.4 +/- 0.6	0.3 +/- 0	0.3 +/- 0	1.1 +/- 0.4			
129	CT	no sample	0	<1	0	0	<1			
	FLI	1.0 +/- 0.6	0	0.3 +/- 0.4	0.5 +/- 0.1	0.1 +/- 0.2	0			
130	CT	0	*0*	*0*	0	*0*	*0*			
ID		Base line	Day 1	Day 2	Day 3	Day 5	Day 7	Day 30	Day 39	Day 42
	FLI	0.7 +/- 0	0							
131	CT	<1	0							
	FLI	0.8 +/- 0.1	0.6 +/- 0.7	0.1 +/- 0.1	6.8+/-0	0.4 +/- 0.6	0.2 +/- 0.3			
132	CT	<1	*0*	*0*	*0*	*0*	*0*			
	FLI	2.1 +/- 0.3	1.8 +/- 0.6	1.7 +/- 0	4.0+/-0					
133	CT	0	0	0	0					
	FLI	0.6 +/- 0.8	0.7 +/- 0.6	0.4 +/- 0.2	0.5 +/- 0.7	40.4 +/- 0				
134	CT	0	0	0	0	0				
	FLI	0	0	3.6 +/-1.0		0.6 +/- 0	0			
135	CT	0	0	0		0	*0*			
	FLI	0.1 +/- 0.2	315.3+/-21							
136	CT	0	0							
137	FLI	0.2 +/- 0.3	0.6 +/- 0.8	0.1 +/- 0	0.4 +/- 0.4	0.4 +/- 0.5				
	CT	0	no sample	0	0	<1				
	FLI	1.0 +/- 0.6		15.9+/-1.1	11.8+/-3.0					
138	CT	0		0	0					
	FLI	3.3 +/- 0.6	194+/-0.5	271+/-17	1.8+/-1.3	3.1+/-0.3	3.6 +/-0.3			
139	CT	0	0	0	0	0	0			

ID		Base line	Day 1	Day 2	Day 3	Day 5	Day 7	Day 30	Day 39	Day 42
	FLI	0.9 +/- 0	0.4 +/- 0.6	51.8+/-1.1	0	0.1 +/- 0.1	1.0 +/- 0			
140	CT	0	*0*	0	*0*	0	0			
	FLI	4.3 +/- 0	2.5 +/- 0.7	3.1 +/- 0.7	2.6 +/- 1.6	651+/-192	2.1 +/-0.5			
141	CT	0	0	0	0	0	0			
	FLI	0.3 +/- 0.4	0.4 +/- 0.3							
142	CT	0	0							
	FLI	0.4 +/- 0.6	0.3 +/- 0.4	0.7 +/- 1.0	1.0 +/- 0.2	0.3 +/- 0.4	1.4+/-0			
143	CT	0	0	0	0	0	0			
	FLI	1.1 +/- 0.3	0.3 +/- 0	0.6 +/- 0.2	0.9 +/- 0.4	0.1 +/- 0.2	0.4 +/- 0.6			
144	CT	0	<1	0	<1	*0*	*0*			
	FLI	1.5 +/- 0.3	0	0.8 +/- 1.1	1.4 +/- 0.3	0.6 +/- 0.8				
145	CT	0	*0*	*0*	0	*0*				
	FLI	0.3 +/- 0	1.1+/-1.1		0.9 +/- 0.8	0.6 +/- 0	0.1+/-0.2			
146	CT	0	0		0	0	0			
	FLI	0.6+/-0.4	0.5+/-0.7	0						
147	CT	0	0	0						
	FLI	0	0.1 +/- 0.2	0.5 +/- 0.5	0.7 +/- 0.6	0.1+/-0.2	0.4+/-0.1			
148	CT	0	0	0	0	0	0			
149	FLI	0.4+/-0.5	0.6 +/- 0	0.4+/-0.6						
	CT	0	0	0						

Dr. Park is in the process of analyzing these data in preparation for the next step of this collaboration.

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ORIGINAL ARTICLE

Immunologic quantitation of tissue factors

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Summary. The large number of conflicting reports on the presence and concentration of circulating tissue factor (TF) in blood generates uncertainties regarding its relevance to hemostasis and association with specific diseases. We believe that the source of these controversies lies in part in the assays used for TF quantitation. We have developed a highly sensitive and specific double monoclonal antibody fluorescence-based immunoassay and integrated it into the Luminex Multi-Analyte Platform. This assay, which uses physiologically relevant standard and appropriate specificity controls, measures TF antigen in recombinant products and natural sources including placenta, plasma, cell lysates and cell membranes. Comparisons of reactivity patterns of various full-length and truncated TFs on an equimolar basis revealed quantitative differences in the immune recognition of TFs by our antibodies in the order of TF 1-263 > 1-242 > 1-218 > placental TF. Despite this differential recognition, all TF species are quantifiable at concentrations $\leqslant$ 2 pM. Using a calibration curve constructed with recombinant TF 1-263 and plasma from healthy individuals ($n = 91$), we observed the concentration of TF antigen in plasma to be substantially lower than that generally reported in the literature: TF antigen in plasma of 72 individuals (79%) was below 2 pM (quantitative limit of our assay); TF antigen levels between 2.0 and 5.0 pM could be detected in six individuals (7%); and in 14% (13 plasmas), the non-specific signal was higher than the specific signal, and thus TF levels could not be determined. These differential recognition patterns affect TF quantitation in plasma and should be considered when evaluating plasma TF-like antigen concentrations.

Keywords: fluorescence immunoassay, placenta, plasma, tissue factor.

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Introduction

The tissue factor (TF)/factor (F) VIIa complex controls hemostatic processes, and contributes to thrombosis-related diseases and cancer [1,2]. The large number of inconsistent reports related to the presence and concentration of TF in blood generates uncertainty regarding the relevance of TF to specific pathological conditions [3–9]. The nature of plasma TF, whether full-length, truncated, alternatively spliced [3], or microparticle associated, remains to be determined.

While multiple studies have proposed associations between TF and cardiovascular disease, inflammation, infection and various malignancies [2,5,10–14], direct correlations between TF antigen levels and specific diseases have been elusive. Reports from several studies show significant overlaps between TF concentrations in the plasma of healthy individuals and subjects with various pathological conditions [3,6,15]. Potential reasons for these discrepancies may reside in the assays used for detection and quantitation of TF antigen. Difficulties may include the use of poly-specific antibodies, the lack of specificity control(s), and the absence of physiologically relevant reference standards.

We have developed a sensitive and specific double monoclonal antibody (mAb) fluorescence-based immunoassay for immune quantitation of TF in blood and tissue milieu. These assays were used to determine patterns of immuno-reactivity for various TF antigens and to assess TF in the plasma of healthy individuals.

Materials and methods

Sources of human TF

Recombinant TFs (rTF) 1-218 (from yeast), 1-242 (from *Escherichia coli*) and 1-263 (from insect cells) were generous gifts from Dr Wolfram Ruf (Scripps Research Institute, La Jolla, CA, USA), Drs Shu Len Liu and Roger Lundblad, Hyland division (Baxter Healthcare Corp, Duart, CA, USA), and Dr Richard Jenny (Haematologic Technologies Inc. Essex Junction, VT, USA) respectively. Hemoliance® RecombiPlasTin and Dade® were purchased from Beckman Coulter (Somerset, NJ, USA) and Dade Behring Inc. (Newark, DE, USA), respectively. Placental TF (Thromborel® S) was a gift from Dr Barrow (Behring Diagnostics Inc.,

Liederbach, Germany). Human monocyte TF was prepared from the THP-1 human monocytic cell line (purchased from ATCC, accession # TIB-202, Manassas, VA, USA). The specificity control for TF from the human monocytic cell line was Balb/c mouse-derived monocytic cell line PU5-1.8 (ATCC TIB-61).

Production and characterization of mAbs

Balb/c inbred mice were immunized with rTF 1-218. Fusion experiments and mAb production were performed as reported previously [16]. Isotypes of mAbs were determined by immunoassay using a kit from Zymed (San Francisco, CA, USA). The influence of mAbs on TF-FVIIa amidolytic activity was determined in a fluorogenic assay [17–19]. rTF 1-242 (0.5 nM), anti-TF mAbs or IgG1 control mAb (0–570 nM), and rFVIIa (2 nM) were incubated for 10 min [20]. The rate of hydrolysis of 50 μM fluorogenic substrate 6-(D-Phe-Pro-Arg)amino-1-naphthalene(*n*-butyl) sulfonamide was measured [17,21]. The effect of mAbs on TF-initiated clotting time (PT) was determined as described previously [22] using placental TF and anti-TF or control mAb (final concentration of 0–86 μg mL^{-1}). Rabbit brain TF (Simplastin, bioMerieux Inc., Durham, NC, USA) was used to determine the species specificity of the inhibition. The immunological characterization of human TF-specific mAbs is summarized in Table 1. Monoclonal Ab affinity for TF 1-218 ranged between 16 and 93 nM. Four clones (5, 16, 18 and 48) were suitable for immunoblotting. Based on affinity, specificity, isotype, protein A binding and immunoblotting capability, IgG1 mAb secreting hybridomas could be divided in to at least two clonally unrelated mAb producing cell lines (5/16/18 and 2). Similarly, IgA secreting cells, are divided in at least two clonally unrelated cell lines (19/29/47 and 48). Two mAb clones 5 and 48 were used for assay development. All mAbs shown in Table 1 inhibit 87%–96% of the FVIIa-TF complex amidolytic activity measured in the fluorogenic assay and 87%–97% of TF activity as assessed by clotting assay.

Plasma TF-like antigen

Freshly frozen citrated plasmas from Red Cross blood bank donors at the Fletcher Allen Health Center, University of Vermont, VT, USA were used for immunoassays of TF. The donors were predominantly Caucasian, reflecting the population in Burlington. Human studies were approved by the Institutional Review Board at the University of Vermont.

Preparation of TF-depleted plasma

Citrated blood was collected from several healthy individuals. Plasma (100 mL) passed through a column containing 0.5 mL of anti-TF-5 mAb-coupled Sepharose (1.5 mg mAb 0.5 mL^{-1} Sepharose). The flow-through was collected and used as TF-immunodepleted plasma.

Purification of TF from placenta and monocyte cell lysates

Placenta TF was purified from crude preparations of placenta (Thromborel® S), which were resuspended in TBS buffer (20 mM Tris, pH 7.4, 150 mM NaCl) containing 0.2% Triton X-100 using Sepharose 4B bound to mAb (clone 5, Table 1). Proteins were eluted using 3 M sodium thiocyanate containing 20 mM CHAPS {3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate; Sigma-Aldrich Co., St Louis, MO, USA}. Following extensive dialysis in HBS (0.15 M NaCl, 0.02 M HEPES, pH 7.4) containing 20 mM CHAPS, TF concentration was determined in our in house ELISA (described below) from a calibration curve constructed using rTF 1-263 as the TF standard.

The human monocytic cell line (THP1) or mouse monocytic cell line (PU5-1.8, specificity control) was grown ($0.5–5 \times 10^6$ mL^{-1}) in the absence or presence of *E. coli*

Table 1 Immunological and functional characteristics of antitissue factor clones

Clone*	Relative affinity, IC_{50} (nM)†	Protein A binding‡	Immunoblot capability§	Isotype¶	Inhibition of clotting time**(%)	Inhibition of TF-VIIa activity (fluorogenic assay)††(%)
2	43	–	–	IgG1, κ	97	87
5	50	–	++++	IgG1, κ	95	94
16	28	–	++++	IgG1, κ	94	96
18	28	–	++++	IgG1, κ	89	96
19	16	–	–	IgA, κ	89	96
29	22	–	–	IgA, κ	90	90
47	16	–	–	IgA, κ	87	90
48	93	++++	++++	IgA, κ	89	94

*Clones were selected from fusion of Balb/c spleens immunized with rTF 1-218.
†Relative affinity was determined by competition assay using rTF 1-218 (MW 30 kDa) as the inhibitor.
‡Protein A (PA) binding capability was assessed by immunoassay in which binding of mAb to immobilized rTF 1-218 was probed with HRP-PA (Sigma).
§Binding of mAb to rTF-1-218 was determined by immunoblotting.
¶Isotyping for each mAb was determined by immunoassay using anti-mouse isotype-specific reagents.
**Inhibition of clotting time was performed using the human placental TF reagent, Thromborel® S in the presence of 50–85 μg mL^{-1} of each mAb.
††In the fluorogenic assay, rTF 1-242 was used. % inhibition is shown for 50 μg mL^{-1} mAb. TF, tissue factor; rTF, recombinant tissue factor.

lipopolysaccharides (LPS, 1 μg mL^{-1}, Sigma) for various times (4–8 h). Cells were collected by centrifugation, viability assessed microscopically using trypan blue dye (Sigma), and TF extracted using TBS-0.2% Triton X-100 (10^7 cell mL^{-1} of extraction buffer overnight at 4 °C, end-over-end mixing). TF was purified by immunoabsorption as described above. Monocyte TF concentration was determined using our in house ELISA.

Enzyme-linked immunosorbent assays (ELISA) of TF

Solid-phase direct binding assays were used to evaluate binding of anti-TF mAbs to TF [23,24]. Purified mAb solutions were added to the wells of microtiter plates (U bottom Falcon plates, Becton Dickinson Labware, NJ, USA) pre-coated with rTF 1-218 or TF 1-263. Binding was probed using horseradish-peroxidase (HRP)-anti-mouse IgG (Sigma).

Competition ELISA was performed in the absence or presence of rTF 1-218 (1-250 nM) as previously described [23,24]. The relative affinity (IC_{50}) is the TF concentration that inhibits 50% of the binding of the mAb to immobilized TF.

For the double mAb ELISA, anti-TF-5 mAb (IgG1, κ, Table 1) was immobilized in the wells of PVC microtiter plate (50 μL $well^{-1}$ of 5 μg mAb mL^{-1} phosphate buffered saline; PBS). Binding of TF was probed with anti-TF-48 mAb (IgA, κ, Table 1) using 10 μg mAb mL^{-1} PBS-1% BSA. Binding of anti-TF-48 mAb was detected by HRP-anti-mouse IgA (5 μg Ab mL^{-1} PBS-1% bovine serum albumin; BSA, ICN/Cappel Biomedicals, Aurora, OH, USA). Figure 1 illustrates that ELISA of equimolar concentrations of rTF 1-263 and purified placental TF are similar.

Concentrations and molecular weight of TFs

Stock concentrations of rTFs were as follow: rTF 1-263 (28.7 μM, in 20 mM CHAPS), 1-242 (3.6 μM, in 1.2% octyl β-D-glucopyranoside), 1-218 (56.2 μM in HEPES buffered saline; HBS) and placental TF (1 μM in 20 mM CHAPS). Placental TF concentration was evaluated using rTF 1-263 as standard and confirmed by analyses of its binding stoichiometry to FVIIa (S. Butenas *et al.*, unpubl. data). Concentrations of rTF 1-263, 1-242 and 1-218 were confirmed by amino acid analyses and absorbance at 280 nm. For amino acid analysis 5–20 μg protein (in 100 μL) was dialyzed in water. Samples were lyophilized and sent for analysis (University of Texas Medical Branch, Protein Chemistry Department, Galveston, TX, USA).

The molecular weights of rTF species 1-218 (29.2 kDa), 1-242 (27.8 kDa), 1-263 (32.7 kDa) and natural placental TF (36.2 kDa) were determined using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF; S. Butenas *et al.*, unpubl. data).

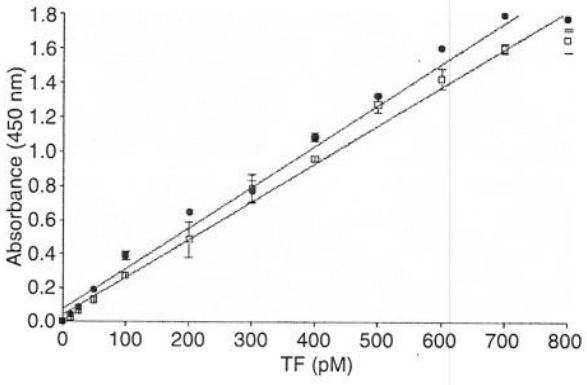

Fig. 1. Calibration curve for recombinant tissue factor (rTF) 1-263 and placental TF by ELISA. Placental TF was affinity purified as described in Materials and methods. Concentration of various dilutions of placental TF was determined from a calibration curve generated with increasing molar concentration of rTF 1-263. Using ELISA, increasing molar concentrations of rTF 1-263 (●) and placental TF (□) are compared. Each point is the mean of duplicate determinations ± SD.

Fluorescence luminex immunoassay (FLI)

Microsphere beads with predefined 658: 712 nm emission ratios (Luminex classification # 035) were coupled to anti-TF-5 mAb according to the manufacturer's instructions (Luminex Corporation, Austin, TX, USA). The binding of TF to mAb-beads was probed with biotinylated anti-TF-48 mAb and detected using R-Phycoerythrin coupled-streptavidin as follows: 50 μL of anti-TF-5 mAb-beads (5×10^3 beads) were added to the wells of a microtiter plate (wells were covered with 1.2 μm PVDF membrane, Multi-Screen filter plates, part # MABV-N1250, Millipore Corporation, Billerica, MA, USA). Fifty microliters of various concentrations of TF in HBS buffer containing 0.1% BSA and 0.2–1% Triton X-100 were added to the beads. Following 30 min incubation at ambient temperature, a vacuum manifold was used to wash away the excess antigen. One hundred microliters of biotinylated anti-TF-48 mAb (10 μg mL^{-1} HBS-1% BSA-0.2% Triton X-100) was added for an additional 30 min. Following three more washes, the beads were reacted with R-Phycoerythrin-streptavidin (100 μL of 5 μg mL^{-1} probe in HBS-1%BSA-0.2% Triton X-100).

Non-specific binding controls were performed by using an isotype-matched mAb with a specificity irrelevant to TF, coupled to microsphere beads with a different 658/712 nm emission ratios (Luminex classification #026) than those used for anti-TF-5. The Luminex technology permits the specific and non-specific binding signal to be determined simultaneously in real time. Data are reported as Δ mean fluorescence intensity units, representing intensity for anti-TF-5 mAb-beads minus that measured for control mAb-beads.

The same FLI assay described above was utilized for detection of monocyte TF. Cells were lyzed (10^7 cells mL^{-1} of TBS buffer with 0.2–1% Triton X-100 as described above) and then 50 μL of various dilutions assayed by FLI described above.

Comparison of the immune-reactivity of TFs using a commercial ELISA kit

We used the IMUBIND TF ELISA kit from American Diagnostica (Stamford, CT, USA) to compare the immune-reactivity of various TFs. The assay was performed according to manufacturer's instructions.

Immunoblot profile of TFs

Immunoblotting was performed as described [25,26] following SDS-gel electrophoresis on 4–12% gradient gels under reducing conditions. The immunoblot standards, Magic-Mark™, were purchased from Invitrogen (Carlsbad, CA, USA).

Flow cytometric analyses

Flow cytometric analyses of membrane-bound TF was performed using THP1 unstimulated cultured monocytes or monocytes stimulated overnight (approximately 16 h) using 1 μg mL^{-1} LPS as described previously [27].

Results

Evaluation of binding specificity of anti-TF mAbs

Figure 2A illustrates that various forms of TF can be detected by immunoblotting using anti-TF mAbs. Placental TF exhibits an SDS apparent molecular mass approximately of 45 kDa while rTF1-263 and rTF 1-218 show an apparent molecular mass approximately of 40 kDa. rTF 1-242 exhibits an apparent molecular mass of 32 kDa. The molecular mass of Innovin TF is similar to that of rTF 1-242, while the mobility pattern of RecombiPlasTin TF is similar to that of rTF 1-263. TF produced by recombinant techniques (rTF 1-218, 1-242 and 1-263) are not equivalent, and none is equivalent to natural placental or monocyte TFs (Fig. 2B). None of the SDS apparent molecular weight (MW) correlates to the true MW of the TF proteins and its use to construct standard curves introduces additional errors. The differences in apparent MW of recombinant and natural TF proteins are likely caused in part by different levels of post-translational modification. Despite this heterogeneity, all TF species react with anti-TF-5 and anti-TF-48 mAbs (Table 1). Because of MW differences, our data are expressed in molar terms.

Flow cytometric analyses were performed to test the ability of the anti-TF-5 mAb to bind to TF displayed on the surface of THP1 monocytes [27]. Prior to LPS stimulation, 0.5% of cells bound Ab compared with 97.3% after 16 h of stimulation. Figure 3 illustrates TF quantitation in crude lysates of unstimulated and stimulated THP1 human-derived monocytic cell lines. The specificity of TF detection in crude lysates of human monocytes was tested, using lysates from mouse monocytic cell line. These results indicate that anti-TF mAbs bind specifically to cell-bound and intracellular human TF.

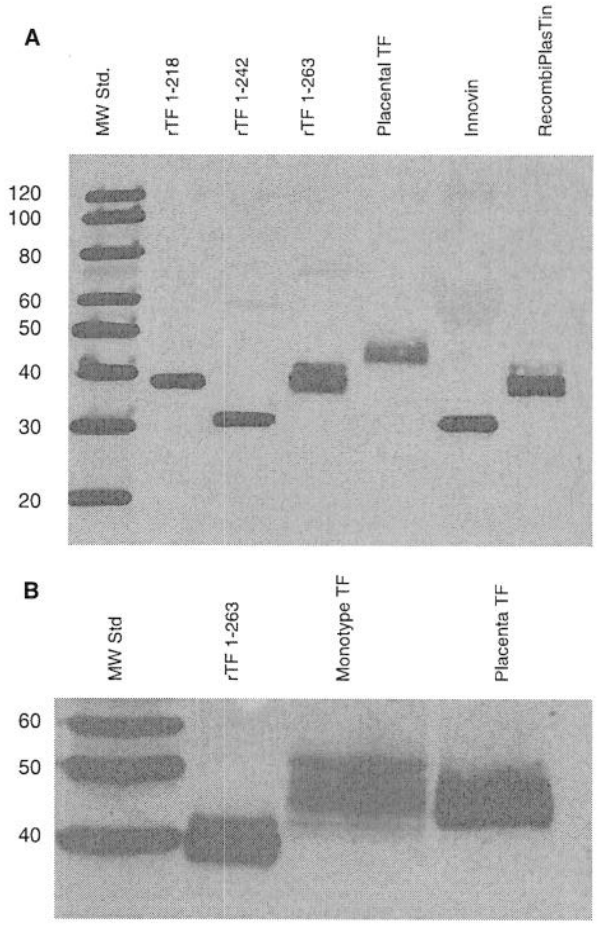

Fig. 2. Immunoblot profiles of recombinant, natural placental and monocyte tissue factor (TF). Proteins (4 ng) were subjected to SDS-PAGE on 4–12% gradient gels under reducing conditions. Proteins were probed with 5 μg mL^{-1} pooled anti-TF-5, anti-TF-18 and anti-TF-48 mAbs (see Table 1), and detected with HRP-sheep anti-mouse IgG and ECL detection reagents. (A) Comparison of placenta TF with rTF; (B) comparison of monocyte and placenta TFs with full-length rTF 1-263.

Evaluation of the sensitivity and specificity of the FLI of TFs

To generate calibration curves for the variety of TFs, molar concentration of each TF species was used based on the MW of TFs obtained by MALDI-TOF (see Materials and methods). The concentration of placental TF was determined by our in-house ELISA (Fig. 1) and confirmed by an analysis of its binding stoichiometry with FVIIa (S. Butenas *et al.*, unpubl. data). By SDS-PAGE, placenta TF appeared 90% pure when compared with rTF 1-218, 1-242 and 1-263.

Using Multi-Analyte Platform (LMAP), the assay specificity and reproducibility of FLI were assessed by spiking various molar concentrations of rTF 1-218 and 1-263 in buffer containing 0.2% Triton X-100 (Fig. 4A). Co-variance (CV%) for six independent experiments performed over 2 months was in the 12.1–13.6% range between 2 and 20 pM (Fig. 4A). Thus quantitative reliable detection limit of the assay was in the 2 pM range as deduced from the CV%. These data indicate

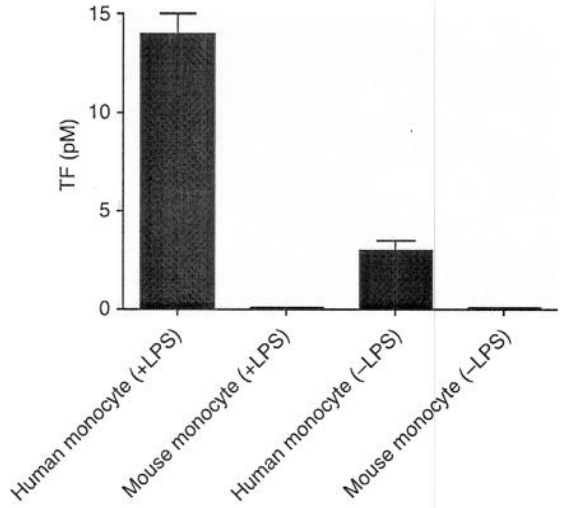

Fig. 3. Quantifying TF antigen in crude cell lysates. THP-1 cells (0.5–1 × 10^6 cells mL^{-1}) were cultured for 4–8 h in the absence or presence of 1 μg mL^{-1} LPS. TF was extracted using TBS-0.2% Triton X-100 and subjected to FLI (see Materials and methods). TF concentration is for cell lysates from 10^6 cells mL^{-1}. Each point is the mean of duplicate determinations ± SD.

that in FLI, using equimolar TF concentrations, the two rTF proteins are recognized differentially in the order of 1-263 > 1-218.

Physiologically relevant calibration curves for determination of plasma TF-like antigen concentrations were more relevant if generated in a plasma milieu with specificity beads included (see Materials and methods). Figure 4B illustrates the immune-reactivity patterns of rTFs 1-218, 1-242, 1-263 and placenta TF in the milieu of TF-depleted plasma. TFs are recognized differently with rTF 1-263 producing the highest signal and placenta TF giving the lowest signal at equimolar concentrations. The quantitative reliability limit of TF analysis in FLI performed in the presence of TF-depleted plasma is ⩽ 2 pM range. Though the fluorescence signal in the presence of plasma proteins is decreased, the quantitative limit remains within 2 pM range for all TF species. Thus, plasma proteins do not compromise the FLI assay sensitivity, specificity and detection concentration. The basis of signal quenching at low concentration in the presence of plasma protein is not clear and may be related to differences inherent of TF proteins with other components of plasma.

Plasma TF-like antigen concentration

Experiments described above demonstrate conclusively that FLI is specific and sensitive and that various TF species ranging from recombinant to natural can be detected and quantified. To determine TF antigen concentrations and ranges in plasma of healthy population, TF antigen was determined for 91 Red Cross blood bank donors (Fig. 5). Seventy-two individuals (79%) had undetectable (< 2 pM) TF antigen levels while in six individuals (7%), TF antigen was detected (2.3–5.0 pM, Fig. 5). In 13 individuals (14%), the non-specific signal was

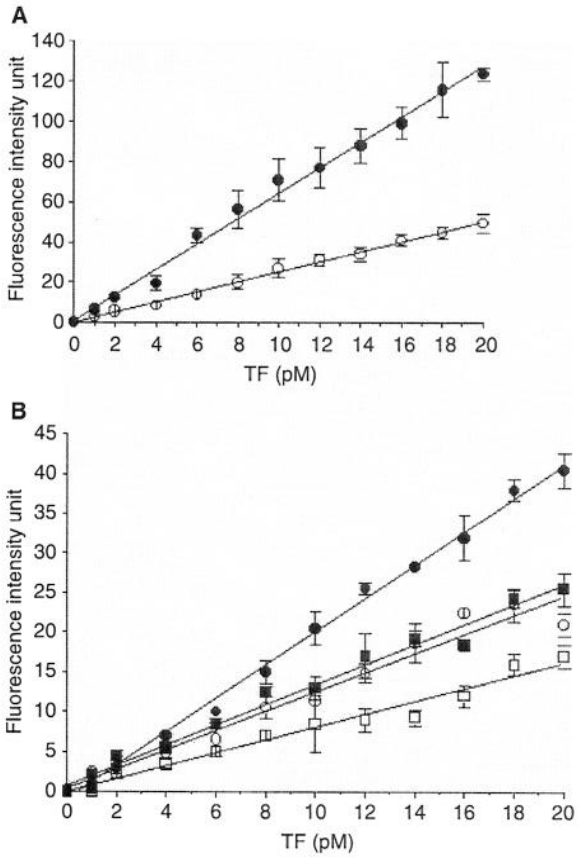

Fig. 4. Fluorescence-based immunoassay of tissue factor (TF; Fluorescence Luminex Immunoassay; FLI). (A) Various molar concentrations (0–20 pM in HEPES buffered saline 0.2% TritonX-100) of recombinant tissue factor (rTF) 1-218 (○), or rTF 1-263 (●) were added to the wells of a microtiter plate containing a mixture of anti-TF-5 mAb-beads and an isotype-matched control mAb-beads (see Materials and methods). Binding was probed with biotinylated anti-TF-48 mAb and R-phycoerythrin-streptavidin. Each point is the mean of 12 independent determinations ± SD. (B) rTF 1-218 (○), 1-242 (■), 1-263 (●) and placenta TF (□) in TF-immunodepleted plasma were added to wells containing anti-TF-5 mAb-coupled- and control-mAb beads as described above.

higher than specific signal leaving the TF concentration unresolved. These results demonstrate that there is TF antigen in plasma, but its concentration is significantly lower than that reported by others using the commercially available IMUBIND kit (see Table 2).

Determination of immune-reactivity patterns of TFs using IMUBIND ELISA kit

Most published data concerning the concentration of circulating TF in plasma have been generated using the IMUBIND kit from American Diagnostica (Greenwich, CT, USA) (Table 2). Calibration curves were generated using the IMUBIND rTF 1-263 standards with established concentrations provided by the manufacturer converted to pM using a molecular mass of 32.7 kDa (see Materials and methods).

Figure 6 illustrates a comparison between the immune-reactivity of the commercial rTF 1-263 calibrator and our

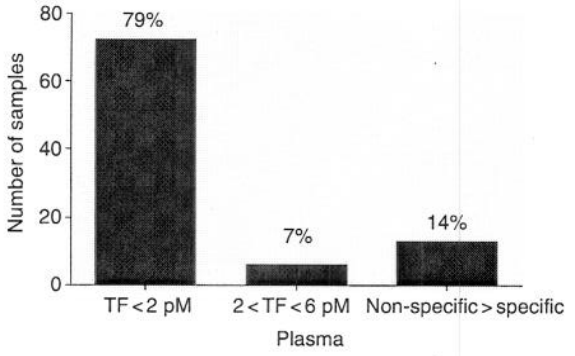

Fig. 5. Tissue factor (TF) antigen concentration in healthy population. Plasmas from 91 individuals were subjected to Fluorescence Luminex Immunoassay as described in Fig. 4. Following subtraction of the non-specific signal (beads coupled to an isotype matched mAb with specificity irrelevant to TF) from specific signal, TF concentrations were determined from a calibration curve of recombinant TF 1-263 (similar to that in Fig. 4B).

Table 2 Reported TF levels in healthy individuals

Plasma TF-like antigen (pg mL^{-1})	TF (pM)	Plasma (*n*)	Reference
135 ± 8	4.1 ± 0.2	?	[52]
61 ± 59*	1.9 ± 1.8 (20.5)	6	[3]
135.4 ± 8.1	4.1 ± 0.2	10	[4]
141 ± 45	4.3 ± 1.4	Not reported	[8]
165 ± 139	5.0 ± 4.2	10	[37]
76 (32–108)	2.3 (1.0–3.3)	21	[7]
142.5 (28–255.3)	4.3 (0.8–7.8)	48	[53]
142 (18–262)	4.3 (0.5–8.0)	103	[6]
102 ± 18.5	3.1 ± 0.6	10	[54]
158 ± 60	4.8 ± 1.8	42	[5]
139 (28–200)	4.2 (0.8–6.1)	28	[55]
135.4 ± 8.1	4.1 ± 0.2	10	[56]
~140 ± 5	4.3 ± 0.1	10	[57]
187.3 ± 108.7	5.7 ± 3.3	22	[9]

*(One control had 672 pg mL^{-1}). TF concentrations were determined using the IMUBIND ELISA kit in which a mAb captures TF and a polyclonal Ab is used to detect TF binding. Concentrations given in pg mL^{-1} were converted to pM using a molecular mass of 32.7 kDa for rTF 1-263.
?, not specified; ~, estimated from graph.

in-house rTF 1-263 and placental TF calibrators using the IMUBIND assay. At equimolar concentrations (Fig. 6), there are differences between the immune-reactivity pattern of the IMUBIND calibrators and our in-house rTF 1-263 calibrator (Fig. 6). In the IMUBIND ELISA, rTF 1-263 and placental TF are recognized equally well (Fig. 6). Quantitative detection limit of the IMUBIND assay in buffer appears to be ⩾ 1.5 pM. The quantitative limit of 0.3 pM (10 pg mL^{-1}) suggested by the manufacturer could not be verified as the lowest calibrator concentration provided in IMUBIND kit is 50 pg mL^{-1} (1.5 pM). The quantitative detection limit of the IMUBIND assay in plasma was determined by spiking increasing concentrations (0, 2, 4, 8, 16 and 32 pM) of rTF 1-263 into TF-depleted plasma and subjecting the spiked plasmas to the IMUBIND ELISA (data not shown). When

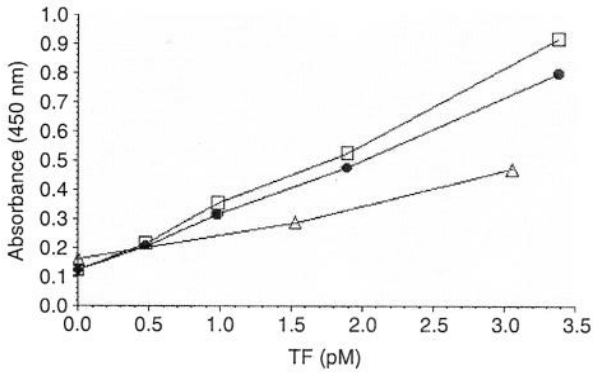

Fig. 6. Comparison of the immune-reactivity pattern of equimolar concentrations of the IMUBIND kit and our inhouse calibrators. Comparison of the low range (0–3.5 pM) IMUBIND calibrator (△), in-house rTF 1-263 (●) and placental TF (□) in buffer using IMUBIND ELISA.

IMUBIND calibrators were used to calculate the data, reliable detection concentration was ⩾ 4 pM. The signal from 2 pM spiked-plasma was too low to enable reliable determination of TF concentration.

TF antigen concentration was determined for several plasmas from Red Cross donors and compared with TF concentrations obtained by FLI (Table 3). To show that the lower concentration of plasma TF-like antigen obtained by FLI is not due to insufficient detergent, 1% Triton X-100 was used. Accordingly, calibrators were generated in 1% Triton X-100. As expected, a higher apparent TF concentration was obtained for individuals by the IMUBIND ELISA when compared with FLI. These results provide at least a partial explanation for higher TF antigen concentration obtained by IMUBIND ELISA. When molar concentration of each calibrator is compared, it appears that the IMUBIND calibrator contains less protein, which results in differences in immune-reactivity of IMMUBIND when compared with our in-house calibrator.

Table 3 Comparison of plasma TF-like antigen concentration by the IMUBIND ELISA and fluorescence luminex immunoassay (FLI)

Individual ID	The IMUBIND ELISA*	FLI†
Plasma TF-like antigen (pM)		
4710	8.4 ± 0.01‡	2.5 ± 1.4
5958	1.9 ± 0.5	0.5 ± 0.7
4934	2.2 ± 0.01	0.5 ± 0
5694	4.5 ± 0.3	1 ± 0
4835	7.5 ± 0.3‡	2.6 ± 2.2
3596	2.7 ± 0.3	1 ± 0
3699	1.9 ± 0.1	2.3 ± 0.8
5339	1.8 ± 0.3	0

*The IMUBIND ELISA was performed in the presence of 0.1% Triton X-100 and 25% plasma as suggested by the manufacturer.
†FLI was performed in the presence of 1% Triton X-100 and 50% plasma.
‡These are the only values for which real calibrator points were available. All other values were read from a hypothetical line that connects 1.5 pM to 0 point (see Fig. 6).

Discussion

TF-specific mAbs and immunoassays described here can quantify TFs (recombinant, placenta, cell lysates and plasma) with high sensitivity and specificity (Figs 2–4). Differential immune-recognition of TFs in FLI (Fig. 4) is not due to differences in TF antigen concentration as TF concentrations were verified by various approaches including absorption at 280 nm, amino acid analysis, ELISA (Fig. 1) and binding stoichiometry. Immunoblotting confirmed that mAbs bind to TF antigens and demonstrated that placental and monocyte TFs are equivalent but different from recombinant full-length (1-263) and truncated TFs (1-242 and 1-218, Fig. 2). Despite heterogeneity among TFs, all were detected by anti-TF mAbs (Figs 2 and 3) and could be quantified in fluorescence immunoassay (Fig. 4). Although placental TF was calibrated by ELISA using a calibration curve generated for increasing molar concentration of rTF 1-263 (Fig. 1), in the highly sensitive FLI assay, there was a difference in the immune recognition of rTF 1-263 and placental TF. This difference is due to high sensitivity of the FLI in which picomolar concentrations of TFs can be detected. Because quantitative reliability limit of ELISA is in sub-nanomolar range, differences in the immune recognition of rTF 1-263 and placental TF could not be readily observed. Despite these variations in recognition, 2 pM of each TF species could be detected in plasma milieu.

Our data also conclude that there is quantifiable TF-like antigen in the plasma of some healthy individuals (Fig. 5) but that the TF concentrations observed are substantially lower than those reported previously by others (an average of 4 pM, Table 2) [9,15,28]. In contrast to low but detectable TF-like antigen concentration in plasma, TF-related activity was not observed in the blood or plasma from healthy individuals [27,29] even though others have reported presence of active TF in plasma with concentrations as high as 37 pM [30].

In addition to controversies surrounding TF activity in blood, there are also disagreements with respect to how much TF antigen actually circulates in blood and whether or not levels of circulating TF antigen correlates with a specific disease. Compilation of data from literature between 1995 and 2006 indicates that the reported TF levels among healthy individuals and patients with various pathological conditions, including cardiovascular diseases, do not show a significant correlation or pattern to enable one to conclude a disease or treatment out come unambiguously [5,6,31,32]. Use of other parameters such as tissue factor pathway inhibitor, prothrombin fragments, FXII, homocysteine, protein C, and markers of inflammation in conjunction with TF antigen levels have not solved this problem [4,6,32,33].

One reason for discrepant results involving TF antigen concentration are immunoassays of TF, which use polyclonal anti-TF antibodies for TF detection. Higher plasma TF-like antigen concentrations have been reported using the IMUBIND ELISA, a commercially available kit that uses a combination of mAb and polyclonal Ab [3,7,15,32,34–42]. Table 2 summarizes the TF concentrations in plasma of healthy individuals by several groups using the IMUBIND assay kit. TF concentrations and ranges vary over a range of 152-fold (2–304 pg mL^{-1} or approximately 0.06–9.3 pM) comparing two different studies [3,37] and 60-fold in a single study [3]. Table 2 demonstrates that reported TF concentration in plasma of healthy individuals is in 4 pM range [excluding the individual with high plasma TF-like antigen (20.5 pM)]. Although FLI exhibits differential recognition of various TF species, TF concentration above 2 pM could have been detected readily for all TF species (1-218, 1-242, 1-263 and placental TF), yet TF concentration in 79% of healthy individuals was below 2 pM.

Comparison of the results of the IMUBIND ELISA with FLI showed that plasma of two patients (4710, 4835, Table 3) contained significantly higher concentration of TF when tested by the IMUBIND ELISA. One possible explanation is that plasma of these two patients contains crossreactive proteins that are recognized by the IMUBIND probes but not by FLI probes. Our results clearly underscore the importance of specificity controls in immunoassays involving human plasma. In 14% of healthy individuals' plasma, the non-specific signal was higher than the specific signal (Fig. 5). None of the studies reported in Table 2 included specificity controls to demonstrate that the observed signals are antigen specific. One major problem with the immunoassays involving human plasma is the interference of heterophilic Abs that can skew the results [43–46]. Thus experiments involving plasma should include specificity controls similar to those reported here.

There are also limited numbers of reports regarding immunoassays of TF that utilize two TF-specific mAbs. For example, using double mAb sandwich assay, Koyoma [47] reported plasma TF-like antigen concentration of approximately 5 pM ($n = 30$), which is substantially higher than values obtained in our studies (< 2 pM in 79%, 2.3–5.5 pM in 7%, Fig. 4). In the absence of specificity controls and data regarding assay's quantitative reliability limit in plasma milieu, it would be difficult to determine whether the signal is antigen specific. In another study, Soejima [48] has used a commercially available kit that uses two TF-specific mAbs. The reported TF antigen levels in heparin treated patients with angina pectoris was 6 ± 0.12 pM ($n = 14$) and in a second group with angina pectoris and chest pain was 5.3 ± 0.18 pM ($n = 9$). The detection limit of this particular assay in the presence of plasma proteins (as referenced by the authors) is reported to be in 1000 pg mL^{-1} (30.6 pM) range [49], thus it appears that the signal was below the detection limit and perhaps noise signal.

The quantitative reliability limit of FLI is in 2 pM range and is comparable to that of the IMUBIND ELISA. van der Putten *et al.* [31] have reported a double mAb ELISA for TF with a detection concentration of 40 fM. However, the calibration curve presented in their manuscript does not support their claim. Apparent lack of sensitivity of their TF immunoassay is supported by the fact that their assay cannot produce any signal with the IMUBIND calibrator. In FLI, the IMUBIND calibrators did produce significant signal but in comparison

with equimolar concentration of our in-house rTF 1-263, the fluorescence signal was significantly lower.

In summary, our data show that anti-TF mAbs (clones 5 and 48) bind plasma, truncated and full-length recombinant, natural and cell-bound TF (Figs 2–5). Anti-TF-5 clone has been shown to bind membrane-bound TF on THP-1 human monocytic cell line and human blood monocytes [27,50,51]. Although we have not tested the binding of these mAbs to alternatively spliced TF [3], we predict that these mAbs would bind to this protein as the extracellular domain of this protein contains most of the extracellular domain of TF [3]. Thus, it is safe to conclude that most known forms of TF can be detected in blood by FLI. In addition, the FLI system reported in this study exhibits the highest level of sensitivity and selectivity as demonstrated by the ability of mAbs to bind TF in buffer and in plasma with minimal change in the quantitative reliable detection concentration (Fig. 4).

The data presented in this manuscript underscore the importance of normalizing epitope presentation among a heterogeneous species of proteins for mAb recognition. This can be achieved through various methods of characterization and evaluation of the TF assay calibrators. These methods include molecular mass determination by reliable techniques, amino acid analysis and the use of molar concentration for protein antigen. The exact nature and function of circulating plasma TF awaits further analyses once large quantities of the circulating plasma TF are purified and characterized. In the mean time a standardized approach should be utilized to minimize variability related to the determination of plasma TF-like antigen antigen.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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Tissue factor activity in whole blood

Saulius Butenas, Beth A. Bouchard, Kathleen E. Brummel-Ziedins, Behnaz Parhami-Seren, and Kenneth G. Mann

Tissue factor (TF) is an integral membrane protein essential for hemostasis. During the past several years, a number of studies have suggested that physiologically active TF circulates in blood at concentrations greater than 30 pM either as a component of blood cells and microparticles or as a soluble plasma protein. In our studies using contact pathway–inhibited blood or plasma containing activated platelets, typically no clot is observed for 20 minutes in the absence of exogenous TF. An inhibitory anti-TF antibody also has no effect on the clotting time in the absence of exogenous TF. The addition of TF to whole blood at a concentration as low as 16 to 20 fM results in pronounced acceleration of clot formation. The presence of potential platelet TF activity was evaluated using ionophore-treated platelets and employing functional and immunoassays. No detectable TF activity or antigen was observed on quiescent or ionophore-stimulated platelets. Similarly, no TF antigen was detected on mononuclear cells in nonstimulated whole blood, whereas in lipopolysaccharide (LPS)–stimulated blood a significant fraction of monocytes express TF. Our data indicate that the concentration of physiologically active TF in non–cytokine-stimulated blood from healthy individuals cannot exceed and is probably lower than 20 fM. (Blood. 2005; 105:2764-2770)

Introduction

Tissue factor (TF) is an integral membrane protein and is an essential component of the factor VIIa-TF complex enzyme, the initiator of blood coagulation in vivo. TF is expressed in numerous tissues of the body and is found in a variety of organs.[1-3] Following mechanical or chemical damage of the vascular wall, extravascular TF is exposed to the blood and binds plasma factor VIIa. TF is also expressed and presented by monocytes and neutrophils following stimulation by inflammatory cytokines.[4-6] Tumor cells also express TF, where TF is related to the metastatic potential.[7-9]

Tissue factor is a 263–amino acid lipoprotein with 3 major domains: (1) an amino-terminal extracellular domain, which binds with high affinity to factor VIIa; (2) a transmembrane domain, which anchors TF to the membrane surface; and (3) a cytoplasmic carboxyterminal domain, which presumably is involved in signal transduction.[10-12] Binding of plasma factor VIIa to membrane TF anchors the proteolytic complex and results in an approximately 2×10^7-fold increase in the enzymatic activity of factor VIIa toward its natural substrates factor IX and factor X.[13]

During the past several years, a number of controversial studies related to the presence, concentration, and functional activity of TF circulating in blood have been published. Several groups of investigators reported the presence of physiologically active TF circulating in blood[14-19] at concentrations as high as 37 pM.[20] It has been reported that this blood-borne TF is located on blood cells and microparticles or circulates as a soluble protein. In contrast, we and several other groups did not observe TF-related activity in blood from healthy humans[21-23] or in mouse blood.[24] Based upon the experience accumulated in our laboratory as well as on reports from other laboratories, blood or plasma activated with (sub)picomolar concentrations of functional TF clots within several minutes.[25-30] These data suggest that physiologically active TF at picomolar concentrations cannot be present in the blood or plasma of healthy individuals in vivo. Similarly conflicting conclusions have been published related to the contribution of blood-borne TF to thrombus growth. Falati et al[31] suggested that vessel wall TF is an essential component of a developing thrombus and did not observe any substantial role for blood-borne TF in thrombus formation. In contrast, Bogdanov et al[32] proposed that soluble blood-borne TF has prothrombotic activity and substantially contributes to thrombus growth.

The current study examines the controversy in quantitative terms regarding the presence and functional activity of blood TF. We used several methods of analysis, including 2 TF activity–sensitive in vitro models of blood coagulation developed in our laboratory[25,26,33,34] and flow cytometry with a well-characterized monoclonal antibody, to detect TF antigen in blood and on monocytes and platelets from healthy individuals.

Materials and methods

Materials

Human coagulation factors VII, X, IX, and prothrombin were isolated from fresh frozen plasma using the general methods of Bajaj et al[35] and were purged of trace contaminants and traces of active enzymes as described.[34] Human factor V and antithrombin-III (AT-III) were isolated from freshly frozen plasma.[36,37] Recombinant factor VIII and recombinant TF (residues 1-242) were gifts from Drs Shu Len Liu and Roger Lundblad (Hyland

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Division, Baxter Healthcare, Duarte, CA). Recombinant human factor VIIa was a gift from Dr Ula Hedner (Novo Nordisk, Bagsvaerd, Denmark). Recombinant full-length TF pathway inhibitor (TFPI) produced in *Escherichia coli* was a gift from Dr K. Johnson (Chiron, Emeryville, CA). Corn trypsin inhibitor (CTI) was isolated from popcorn as described elsewhere.[26] Washed platelets were prepared by the procedure of Mustard et al.[38] Preparation of the TF/lipid reagent was done as described elsewhere.[26] PS (1,2-dioleolyl-*sn*-glycero-3-phospho-L-serine) and PC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine) were purchased from Avanti Polar Lipids (Alabaster, AL) and Ca^{2+} ionophore A23187 was purchased from EMD Biosciences (San Diego, CA). EDTA (ethylenediaminetetraacetic acid), HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid), IgG-FITC (fluorescein isothiocyanate–conjugated goat antimouse immunoglobulin G), and Arg-Gly-Asp-Ser (RGDS) tetrapeptide were purchased from Sigma (St Louis, MO). Phospholipid vesicles (PCPSs) composed of 25% PS and 75% PC were prepared as described.[39] The chromogenic thrombin substrate Spectrozyme TH was purchased from American Diagnostica (Greenwich, CT). D-Phe-Pro-ArgCH_2Cl (FPRck), monoclonal anti-TF (α–TF-5), and anti–factor XI (α–FXI-2) antibodies were produced in house. Citrated plasma was purchased from George King Bio-Medical (Overland Park, KS), α–P-selectin–PE (phycoerythrin-conjugated anti–P-selectin antibody) was purchased from BD Biosciences (San Diego, CA), and Optilyse C was purchased from Coulter Immunotech (Marseille, France). The human monocyte cell line TIB 202 was purchased from American Type Culture Collection (ATCC; Rockville, MD) and low-endotoxin bovine serum albumin was from Irvine Scientific (Santa Ana, CA).

Whole-blood clotting

Eleven healthy donors (male and female; age range, 22-55 years) were recruited and advised according to a protocol approved by the University of Vermont Human Studies Committee and their consent was obtained. All individuals exhibited normal values for the parameters of blood coagulation, plasma protein levels, and platelet count. Experiments were performed in tubes placed on a rocking table enclosed in a 37°C temperature-controlled glove box using fresh blood. For 2 individuals (nos. 1 and 6; Table 1), blood was drawn by venipuncture and immediately delivered (1.0 mL) into the tubes loaded with 0.1 mg/mL CTI and various concentrations (0-10 pM; in duplicates) of relipidated TF (PCPS/TF = 5000) in HBS (20 mM HEPES; 150 mM NaCl, pH 7.4) and 2 mM $CaCl_2$. In all experiments, no more than 35 μL of reagent was loaded in each tube. The clotting time was observed visually by 2 observers and was called when "clumps" were noticed on the side of the tube. For the rest of the individuals, clotting time of CTI blood in the absence of exogenous TF was evaluated.

Plasma clotting

Calcium ionophore A23187–treated washed platelets (fresh or frozen) from individual no. 2 (Table 1) were added at a concentration of 2×10^8/mL to citrated pooled plasma containing 0.1 mg/mL α–FXI-2 or CTI and varying amounts of relipidated TF. Plasma clotting was initiated with 25 mM $CaCl_2$. Clotting times were determined using the ST4 clotting instrument (Diagnostica Stago, Parsippany, NJ).

Table 1. Clotting times of CTI-inhibited fresh whole blood

Individual	Sex	Age, y	Clotting time, s
1	M	24	1220
2	F	24	1225
3	M	55	1275
4	M	23	1380
5	M	22	1395
6	M	52	1500
7	M	24	1545
8	M	25	2010
9	F	24	2025
10	M	25	2700
11	M	53	2940

Synthetic plasma model

Procofactor solution. Washed platelets (4×10^8/mL) from individual no. 8 (Table 1) were incubated in HBS with 2 mM $CaCl_2$ for 10 minutes at 37°C. When desired, 20 pM relipidated TF (molar ratio, PCPS/TF = 5000), 10 μM A23187, and 0.2 mg/mL α–TF-5 were added. Factor V (40 nM) and factor VIII (1.4 nM) were added prior to the initiation of the reaction.

Zymogen-inhibitor solution. Prothrombin (2.8 μM) and coagulation factors VII (20 nM), VIIa (0.2 nM), X (340 nM), IX (180 nM), and XI (60 nM), TFPI (5 nM), and AT-III (6.8 μM) were warmed in HBS with 2 mM $CaCl_2$ at 37°C for 3 minutes. When desired, factors VII and VIIa were omitted.

The reaction was initiated by mixing equal volumes of both solutions, resulting in physiologic concentrations of the zymogens, procofactors, inhibitors, and platelets, 10 pM TF, and 0.1 mg/mL α–TF-5. Following initiation of the reaction, 10-μL aliquots were withdrawn at 1 to 10-, 12-, and 14-minute time points from the reaction mixture; quenched in 20 mM EDTA in HBS (pH 7.4) containing 0.2 mM Spectrozyme TH; and assayed immediately for thrombin activity. The hydrolysis of the substrate was monitored by the change in absorbance at 405 nm using a Molecular Devices V_{max} spectrophotometer (Sunnyvale, CA). Thrombin generation was calculated from a standard curve prepared by serial dilutions of α-thrombin.

Factor X activation

Factor VIIa (0.5 nM) was incubated in HBS with 2 mM $CaCl_2$ (pH 7.4) for 10 minutes at 37°C with either 6×10^8/mL A23187-treated platelets from a single donor or 20 pM relipidated TF in the presence of 100 μM PCPS. Factor X at 170 nM concentration was added and 20-μL aliquots were withdrawn at selected time points (0-5 minutes) and quenched into 20 mM EDTA in HBS (pH 7.4) containing 0.2 mM Spectrozyme Xa. The hydrolysis of the substrate was monitored by the change in absorbance at 405 nm using a Molecular Devices V_{max} spectrophotometer. Factor Xa generation was calculated from a standard curve prepared by serial dilutions of purified factor Xa.

Flow cytometry

Monocyte stimulation. TIB 202 cells (5×10^6/mL) were cultured in serum-free media containing 0.35% low-endotoxin bovine serum albumin in the presence of 1 μg/mL lipopolysaccharide (LPS). Following overnight culture (~16 hours), the cells were removed from the tissue culture plate by gentle trituration, aliquoted (~3×10^5 cells/tube), and washed 2 times by centrifugation (400*g*, 10 minutes) followed by resuspension in HBS. The cell pellets were resuspended in immunostaining reaction mixtures (100 μL final volume) containing 10 μg/mL human Fc fragment and 0.5 μM α–TF-5. Nonspecific antibody binding was assessed by incubating with an isotype-matched irrelevant antibody at the same concentration. Following a 30-minute incubation at ambient temperature, HBS was added and the cells were subjected to centrifugation. The cell pellets were washed one time with HBS by centrifugation and resuspended with 100 μL of a 1:100 dilution of IgG-FITC. Following a 30-minute incubation at ambient temperature, HBS was added and the cells were subjected to centrifugation (400*g*, 10 min). The cell pellets were resuspended with 1 mL 2% paraformaldehyde and stored at 4°C until flow cytometric analyses.

Whole blood. Whole blood (donor no. 6; Table 1) containing 0.1 mg/mL CTI was incubated with Optilyse C (1:1 dilution) to fix the cells and lyse the red blood cells. In other experiments, CTI-containing blood was incubated with 50 U/mL heparin and 100 ng/mL LPS (lipopolysaccharide) for 2 hours and then diluted 1:1 with Optilyse C. Following a 15-minute incubation with Optilyse C, blood was incubated with α–TF-5 (75 μg/mL) or an isotype-matched irrelevant mouse IgG for 30 minutes. The cells were washed by centrifugation followed by resuspension in HBS. Following centrifugation, pellets were resuspended in 100 μL of IgG-FITC (1:100 dilution) and incubated for 30 minutes. The cells were diluted 1:1 with Optilyse C. Following a 15-minute incubation, 300 μL HBS was added and cells were stored at 4°C until flow cytometric analyses.

Isolated blood-cell populations. Human mononuclear cells and platelets were isolated from whole blood of donor no. 6 (Table 1) using standard

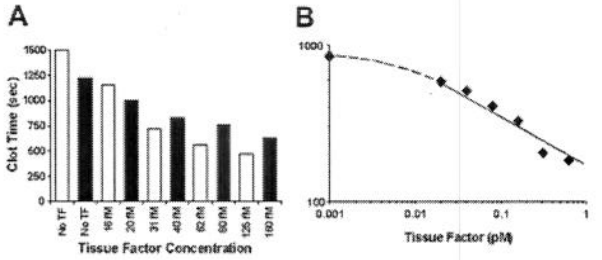

Figure 1. Titration of relipidated TF in CTI-inhibited whole blood and plasma. Increasing concentrations of relipidated TF (TF/PCPS, 1:5000) were added to whole blood containing 0.1 mg/mL CTI (A) or PFP reconstituted with A23187 (a calcium ionophore)–treated platelets at 2 × 10^8/mL (B). ■ represents individual no. 1 (Table 1) and □ represents individual no. 6. Clotting time was determined either visually (whole blood) or using the ST4 clotting instrument (plasma).

techniques.[38] Following appropriate stimulation, cells were immunostained for flow cytometry as described in "Whole blood." To verify platelet activation, A23187-treated platelets were incubated with α–P-selectin–PE and analyzed by flow cytometry. Cells (10 000) were analyzed by flow cytometry on a Coulter EPICS Elite flow cytometer (Coulter, Hialeah, FL). Monocytes and platelets were identified by their forward and side scatter. The positive gate was set such that at least 98% of the cells stained with the irrelevant antibody were negative. Microparticles were defined as having the same side scatter but a smaller forward scatter as the cells from which they were derived.

Results

TF-related activity in whole blood and plasma

To establish if functionally active blood-borne TF is present in healthy individuals, specific experiments were designed to identify TF-related activity in blood and plasma. To prevent the influence of contact pathway–initiated coagulation, 0.1 mg/mL CTI (inhibits factor XI activation by factor XIIa[40]) was added to whole blood. In the presence of CTI, fresh nonanticoagulated blood kept at 37°C with mixing does not clot for more than 1200 seconds in the absence of exogenous TF[27] (Figure 1A). The addition of as little as 16 to 20 fM TF to CTI blood resulted in accelerated clot formation (ie, this extremely low concentration of functionally active TF shortened clotting time by 220 s for donor no. 1 [Table 1; Figure 1A ■] and by 350 s for donor no. 6 [Figure 1A □]). Titrations of TF in CTI blood resulted in shortening of the clotting time in a TF concentration–dependent manner. At the highest concentration of exogenous TF tested (10 pM), whole blood from a healthy individual clotted in 140 seconds. For all 11 individuals tested (male and female; age range, 22-55 years), the clotting time of contact pathway–inhibited blood in the absence of exogenous TF was more than 1200 seconds (20 minutes), extending in some experiments beyond 2400 seconds (40 minutes; donor nos. 10 and 11 in Table 1), suggesting that no detectible concentrations of active TF are present in the blood of healthy donors.

Platelet-free citrated plasma (PFP) from healthy individuals, as well as PFP reconstituted with washed platelets (2 × 10^8/mL), was also tested for the presence of TF activity. The activation of contact pathway coagulation was inhibited by the addition of 0.1 mg/mL CTI or antibody α–FXI-2. Under these conditions, no clot formation was observed in 1000 seconds (the upper time limit for the clotting apparatus ST-4).

To address the question of whether platelet activation induces expression/exposure of functional TF, Ca^{2+} ionophore A23187–treated washed platelets were added to PFP at the mean physiologic concentration (2 × 10^8/mL) and the clotting time was measured in the presence or absence of an inhibitory antibody (α–TF-5). In the absence of exogenous TF and α–TF-5, activated platelets and CTI-containing plasma mixture clotted in 835 seconds. The addition of α–TF-5 at 0.1 mg/mL concentration to this plasma had no effect on the clotting time, suggesting that no detectable amounts of functional TF are present on the activated platelet. To test the efficiency of the antibody against TF, α–TF-5 was added to 5 pM relipidated TF, 50 μM PCPS, and 0.1 mg/mL CTI-containing PFP. The clotting time in the absence of α–TF-5 was 190 seconds and in the presence of α–TF-5 the clotting time was more than 1000 seconds.

Relipidated TF was further titrated into activated platelet-containing plasma (Figure 1B). As mentioned in the previous paragraph, in the absence of exogenous TF, CTI-containing plasma clotted in 835 seconds. The addition of exogenous TF caused decreases in the clotting time in a TF concentration–dependent manner. At as low as 20 fM TF, the clotting time decreased to 582 seconds. At the highest TF concentration tested (5 pM), a 106-second clotting time was observed (data not shown).

One must conclude from the results of whole blood and plasma clotting experiments that the presence of functionally active TF at the high concentrations previously reported to be circulating in healthy individuals would lead to a massive thrombin generation. The coagulation response to concentrations of TF as low as 20 fM leads to the conclusion that this concentration of functional TF in nonactivated blood and activated platelets from healthy individuals is well beyond the upper limit of potential active TF concentration in blood.

Platelet-related TF activity

The potential of platelet-related TF activity was evaluated using synthetic plasma composed of all known elements of the extrinsic pathway (Figure 2A). Resting platelets added to the synthetic plasma did not initiate detectable levels of thrombin generation (Figure 2A ○). With A23187-treated platelets, thrombin was produced at a maximum rate of 3.1 nM/second and reached 265 nM (Figure 2A ▲). TF per se has no proteolytic activity, it only enhances that of factor VIIa toward its substrates, both natural and synthetic.[13,41] Thus, if the observed thrombin generation in the presence of activated platelets is related to TF, the omission of factor VIIa and factor VII (as possible sources of factor VIIa) from the reaction mixture should prevent thrombin generation. However, neither the initiation phase of thrombin generation (4 minutes) nor the maximum rate was substantially affected by the

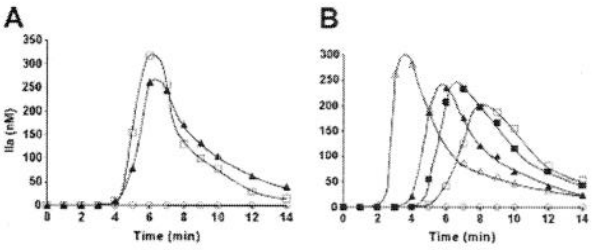

Figure 2. Thrombin generation in synthetic plasma. Synthetic plasma was composed of factors V, VIII, VII, VIIa, IX, X, and XI, prothrombin, TFPI, and AT-III at their mean physiologic concentration. (A) Thrombin generation was initiated with 2 × 10^8/mL A23187-treated platelets in the absence of TF and either in the presence (▲) or in the absence (□) of factors VII and VIIa. (B) Thrombin generation was initiated either with 2 × 10^8/mL A23187-treated platelets in the absence (▲) or presence of 0.1 mg/mL α–TF-5 (■) or with TF in the presence of 2 × 10^8/mL resting platelets and either in the absence (△) or presence of α–TF-5 (□). ○ represents thrombin generation in the presence of resting platelets and absence of TF.

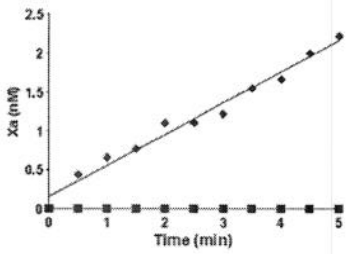

Figure 3. Factor X activation by the extrinsic Xase (◆) and A23187-treated platelets (■). Either treated platelets at 6×10^8/mL concentration ($3 \times$ mean physiologic) or 20 pM relipidated TF were incubated with 0.5 nM factor VIIa in the presence of 100 μM PCPS. Factor X (170 nM) was added and factor Xa generation was measured in a chromogenic assay.

absence of factors VII and VIIa (Figure 2A □). Thus the active agent was not TF.

In a further experiment, the influence of α–TF-5 on thrombin generation initiated either with relipidated TF or with A23187-treated platelets was compared (Figure 2B). In the absence of TF, no thrombin generation was observed in the presence of resting nonactivated platelets (Figure 2B ○). The addition of 10 pM relipidated TF in the presence of resting platelets caused rapid thrombin generation at a rate of 4.9 nM/second after an initiation phase of 2 minutes (Figure 2B △). For the same reaction conditions, the addition of α–TF-5 prolonged the initiation phase (to 5 minutes) and suppressed the maximum rate of thrombin generation to 1.4 nM/seconds (Figure 2B □). The maximum level of active thrombin formed was also reduced from 300 nM in the absence of antibody to 200 nM in the presence of antibody. The addition of A23187-treated platelets to synthetic plasma in the absence of exogenous TF (Figure 2B closed symbols) led to thrombin generation at a maximum rate of 2.7 nM/second (Figure 2B ▲). The initiation phase observed was approximately 4 minutes, and the maximum level of active thrombin formed in the reaction was 250 nM. The addition of 0.1 mg/mL α–TF-5 to the synthetic plasma with A23187-treated platelets had almost no effect on the maximum rate or level of thrombin generation (Figure 2B ■). The initiation phase duration was slightly prolonged (by < 1 minute).

A proteolytic activity leading to thrombin generation was observed in the synthetic plasma in the presence of A23187-treated platelets. Although the data presented in Figure 2 suggest that this activity is not related to TF, a more specific reaction system was used to evaluate the ability of ionophore-treated platelets to enhance factor VIIa activity toward its natural substrate factor X. Factor VIIa and factor X were added to A23187-treated platelets at $3 \times$ their mean physiologic concentration (6×10^8/mL). No factor Xa generation was observed over 5 minutes of the reaction (Figure 3 ■). In a control experiment performed under the same conditions with 20 pM relipidated TF, factor X was activated at a rate of 0.4 nM/minute (Figure 3 ◆).

The data shown in Figures 2 and 3 lead to the conclusion that the ability of A23187-treated platelets to initiate thrombin generation in the synthetic plasma mixture is most likely not provided by TF.

The search for TF antigen in whole blood and on purified cells

Whole blood. The majority of reports related to blood-borne TF use immunochemical methods employing monoclonal antibodies, which recognize epitope(s) on the TF molecule whether or not it is functionally active. We evaluated if TF antigen could be detected on blood cells using flow cytometry. As a negative control, cultured LPS-activated monocytes were stained using an irrelevant isotype-matched antibody (Figure 4A). As a positive control, LPS-activated monocytes were stained with α–TF-5. After a 12-hour stimulation with 1 μg/mL LPS, 98% of monocytes stained positively for TF (Figure 4B). Additionally, the majority of microparticles generated during the preparation and immunocytochemical staining of the LPS-stimulated monocytes were TF positive (Figure 4C).

Purified cells. The monocytes (Figure 5A) and platelets (Figure 5B) present in unstimulated whole blood expressed no detectable TF. When whole blood was stimulated with 0.1 μg/mL LPS for 2 hours, 14% of monocytes were positive for TF (Figure 5C). LPS had no effect on the expression of TF by platelets (Figure 5D). To answer the question if TF antigen is expressed/exposed on activated platelets, flow cytometric analyses were performed on A23187-treated platelets (Figure 6). No TF was detected on either resting (Figure 6A) or treated platelets (Figure 6B). Under these conditions, approximately 91% of the platelets were positive for the platelet activation–dependent surface protein P-selectin (Figure 6C).

Discussion

The conflicting reports related to the presence, source, and function of blood-borne tissue factor[16,17,20,21,31,32,42-44] stimulated the current study. We looked for TF-related activity and antigen in blood, plasma, and platelets. The data presented in this study indicate that less than 20 fM functionally active TF can be present in unstimulated blood and plasma from healthy individuals. In contrast, immunoassay showed that this plasma contains approximately 0.5 pM TF-like antigen.[45] No TF-related activity was observed using activated platelets either in the clotting, the synthetic plasma, or the extrinsic Xase assays, which allow for the detection of TF at (sub)picomolar concentrations.

TF antigen was not detected on blood mononuclear cells in the absence of intentional LPS stimulation. In contrast to previous studies,[16,43] no TF antigen was detected on platelets present in unstimulated and LPS-stimulated blood or on washed and activated platelets. These observations, as well as those previously published

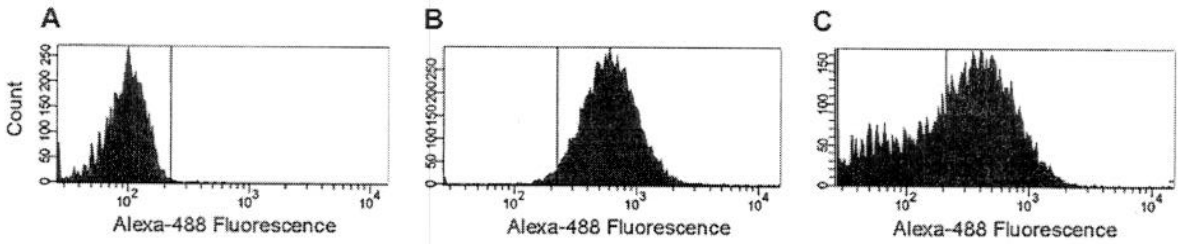

Figure 4. Flow cytometric analyses of cultured LPS-stimulated monocytes and microparticles. Cells (5×10^6/mL) were stimulated overnight with 1 μg/mL LPS. Cells were immunostained either with an irrelevant isotype-matched mouse IgG (A) or with α–TF-5 (B). Panel C shows microparticles generated during the preparation and immunocytochemical staining of monocytes with α–TF-5.

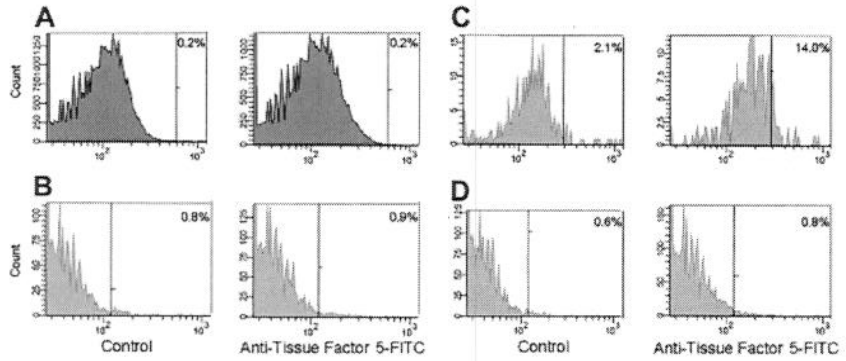

Figure 5. Flow cytometric analyses of monocytes and platelets from whole blood. Monocytes (A) and platelets (B) in unstimulated blood. Monocytes (C) and platelets (D) in LPS-stimulated blood. α–TF-5 was used for immunostaining. The positive analysis regions (vertical bars) were defined such that less than 99% of the cells stained with an irrelevant, isotype-matched mouse IgG were positive. The percentages shown are the percent of positive cells in these analysis regions.

by our[23,25,26,33,34] and several other laboratories,[21,46] suggest an absence of measurable amounts of active TF in blood and plasma from healthy individuals (> 20 fM) and are in contrast to other studies indicating the presence of picomolar amounts of TF in plasma.[14-20]

The origins of the discrepancies in detection of blood-borne TF are of interest. The methods used for the quantification of TF in blood and plasma can be divided into 2 groups: (1) immunoassays and (2) activity-based assays. The first group of assays is based upon the recognition of specific TF epitopes by anti-TF antibodies.[16,18,31,47-49] Although routinely monoclonal antibodies are used in immunoassays of TF, it is likely that these antibodies will recognize not only full-length functional protein but also truncated and inactive forms including TF degradation products.[50] It is not likely that these soluble TF fragments or product are functionally active. Additionally, it is also possible that the antibodies used were not entirely specific for TF antigen and can cross-react with other proteins. These considerations may explain an apparent discrepancy between the levels of TF-like antigen detected in plasma from healthy individuals by the immunoassay and the absence of functional TF activity in the same plasma.

The most commonly used TF activity–based assay evaluates factor Xa generation in the presence of factor VIIa.[17,44,51-53] In these assays, supraphysiologic concentrations of factor VIIa are used, frequently exceeding those circulating in vivo by 2 orders of magnitude.[52,53] At these high factor VIIa concentrations, the soluble form of TF (an extracellular domain of TF) will bind factor VIIa and display a limited proteolytic activity. At a physiologic factor VIIa concentration (~ 0.1 nM), however, the soluble form of TF displays a negligible activity[54] and is not likely to trigger blood coagulation.

It is important to note that there is a large discrepancy between the reported plasma concentration of soluble TF and that required to achieve very limited clotting activity as described by Bogdanov et al.[32] The reported concentration of alternatively spliced TF (soluble form of TF) in plasma is approximately 0.5 pM (consistent with our antigen measurements[45]). In that study, approximately 40 nM of this TF form was used to decrease the plasma clotting time from 233 to 150 seconds (ie, 80 000-fold higher than the reported physiologic concentration). The clotting activity of this TF species is similar to that observed for the extracellular domain of TF.[54] A similar effect on the clotting time was observed using only 2 pM transmembrane domain–containing TF (Figure 1B).[54] Thus, the soluble form of TF was used at concentrations exceeding those reported as present in vivo by almost 5 orders of magnitude to show a limited effect on clot formation.

The suggestion that soluble blood-borne TF has an effect on the pathology of coronary arteries when recruited to thrombi is speculative.[32] There are no data indicating that soluble TF accumulated in thrombi has functional activity; it might be hypothesized that this form of TF can act as an inhibitor of coagulation because by binding factor VIIa, soluble TF will form an inactive complex enzyme. As a consequence, the concentration of factor VIIa available for the complex formation with the functional, membrane-bound full-length TF will decrease.

In a publication by Falati et al,[55] the authors report accumulation of blood-borne (microparticle) TF in the platelet thrombus and suggest that this TF triggers the initiation of blood coagulation. Based upon the data presented in the current study, the concentration of the functionally active TF in blood of healthy individuals cannot exceed a few fM, whereas 5 pM active TF is required to provide approximately 5-minute clotting time in the contact pathway–inhibited whole blood.[27] This clotting time is similar to

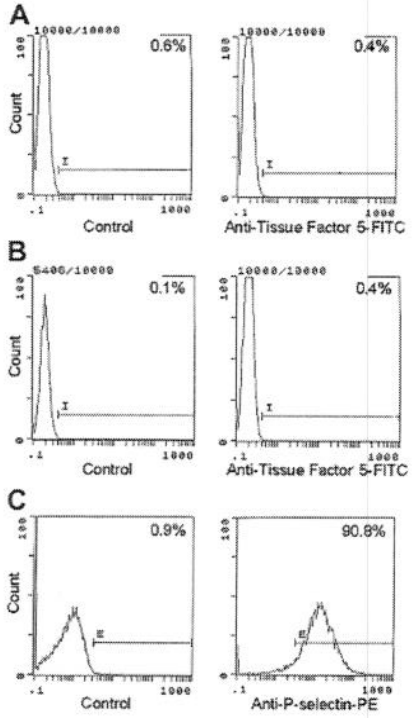

Figure 6. Flow cytometric analyses of resting and A23187-treated platelets. Resting (A) or A23187-treated (B) platelets were immunostained with α–TF-5. A23187-treated platelets were also treated with an anti–P-selectin antibody (C). The positive analysis regions (horizontal bars) were defined such that less than 99% of the cells stained with an irrelevant isotype-matched mouse IgG were positive. The percentage of positive cells in each of these analysis regions is shown.

that observed in the "Simplate" bleeding assay.[56] Thus, the concentration of the blood-borne TF at the site of the vascular injury would have to increase by approximately 3 orders of magnitude to provide normal hemostasis. As a consequence, a life-threatening blood loss would occur during the time required to reach such a blood-borne TF concentration. Additionally, it is possible that the observed blood-borne TF antigen was generated by mechanical injuries inflicted on the mice during the preparations of the animals for the experiment.[55]

A TF-independent initiation of thrombin generation by activated platelets was observed in both synthetic and citrated plasma. This platelet-related initiating activity was less pronounced in citrated plasma (~ 14-minute clotting time) than in the synthetic plasma (~ 4-minute "clotting" time). The difference in activity in 2 reaction systems is caused, most likely, by the absence of natural serine protease inhibitors (α_1-antitrypsin, α_2-macroglobulin, heparin cofactor II, etc) in the synthetic plasma, whereas these inhibitors are present in citrated plasma. The most probable initiator of thrombin generation in these 2 reaction systems is the factor XI–like protein reported to be present in platelets and released in an active form during platelet activation.[57] In a previously published study, a pronounced effect of this activity on thrombin generation in the synthetic plasma was observed.[58] Additionally, upon activation, platelets expose membrane binding sites for the enzymatic reactions leading to thrombin generation and, as a consequence, accelerate that process.[59] A different activity of ionophore-treated platelets in citrated and synthetic plasma allows an assumption that the observed activity in the former reaction system is mostly related to the exposed membrane binding sites, whereas in the synthetic plasma the prevailing source of activity could be related to the factor XI–like protein. An experimental confirmation of the hypotheses presented in this paragraph is a subject of future studies.

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